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Applications of flow cytometry in molluscan immunology: Current status and trends

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ABSTRACT

Flow cytometry (FCM) is routinely used in fundamental and applied research, clinical practice, and clinical trials. In the last three decades, this technique has also become a routine tool used in immunological studies of molluscs to analyse physical and chemical characteristics of haemocytes. Here, we briefly review the current implementation of FCM in the field of molluscan immunology. These applications cover a diverse range of practices from straightforward total cell counts and cell viability to characterize cell subpopulations, and further extend to analyses of DNA content, phagocytosis, oxidative stress and apoptosis. The challenges and prospects of FCM applications in immunological studies of molluscs are also discussed.

1. Introduction

FCM is an extremely powerful method for cell analysis that is fast, accurate, simple to use and can achieve simultaneous measurement of multiple cellular parameters. Modern flow cytometers have been around for over 70 years since the first impedance-based flow cytometry device with an electronic cell volume calculator patented in 1953 [1]. Recent advances in instrumentation, software and fluorochrome chemistry have led to the emergence of FCM applications in a number of fields, including haematology [2], food industry [3], virology [4], pathology [5], plant biology [6], marine biology [7], molecular biology [8] and immunology [9,10].

In immunological studies, FCM is a standard laboratory tool used for both fundamental and applied research, especially for clinal studies [9–11]. Immunologists use FCM to enumerate specific cell subpopulations and measure a diverse number of cytometric parameters, such as membrane surface and intracellular characteristics, cell death, phagocytosis, autophagy, mRNA, transcription factors, signal transduction pathways, lymphocyte metabolism [10,11]. In molluscan immunological research, FCM has been applied to investigate haemocytes since the 1990s, but mostly in bivalve species [12–16]. The applications of FCM for gastropods mainly emerged in the 2000s [17,18]. In these early applications, FCM was primarily used for identification of cell populations and viability [12,13,16]. Currently, the use of FCM has been expanded to multiple parameters of molluscan immunology, including cell count and viability, cell types, phagocytosis, oxidative stress, apoptosis, DNA and protein content. However, the number of FCM parameters used in immunological studies of molluscan species are limited compared to those used in vertebrate immunology.

This contribution highlights the emerging applications of FCM in immunological studies of molluscs for morphological and functional analyses of haemocytes. In addition, we highlight the challenges of using FCM and perspectives for the future development and application of this tool in molluscan immunology.

2. Overview of flow cytometry

FCM is a laser-based technique that is used to analyse the physical and chemical characteristics of cells or particles in a heterogeneous fluid mixture as they pass through a light source. In principle, cell components are stained with fluorescently labelled dyes, so that the quantity of a particular cell component is calculated based on the fluorescence intensity. When the suspensions of cells are injected into the flow cytometer, cell components are excited by the laser, which emits light in a band of wavelengths. Hence, the fluorescence intensity is measured for each particular cell at the rate of thousands of particles per second (Fig. 1). This allows rapid and quantitative analysis of cells in a heterogeneous fluid mixture.

FCM analyses are conducted with different flow cytometric instruments that normally consist of four core components: an illumination source, a fluidic system, an optical bench, electronics and a computer control system [19,20]. In brief, the fluidic system transports stained

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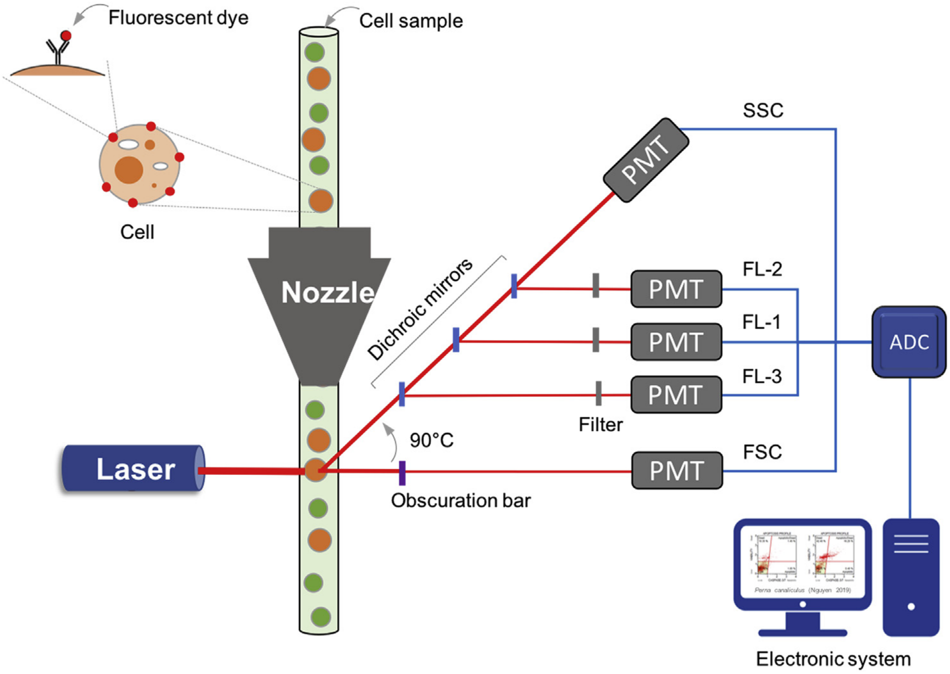

Fig. 1. Schematic diagram of a flow cytometer.
Abbreviations: SSC, side scatter; FSC, forward scatter; PMT, photomultiplier; ADC, analog-to-digital converter; FL, fluorescence.

cells from a suspension into the laser intercept (light beam) in a single file for laser interrogation by one or more light sources. These light sources generate light signals at a specific frequency which are collected, filtered and directed by the optical system to photodetectors. The photodetectors, in turn, measure scatter light signals and convert them into electronic signals which are converted by the electronics system to data for storage, visualization and subsequent analysis by software [20] (Fig. 1). There are two measurements of light scatter by two optical detectors, including forward scatter (FSC) and side scatter (SSC). FSC scatters along the path of the laser which allows identification of cells by size. The measurement of SSC is at a 90° angle relative to the laser which is helpful for identification of the internal complexity (e.g., granularity) of a cell. The combination of FSC and SSC allows physical sorting a heterogeneous cell mixtures into different populations in specialized flow cytometers with sorting capabilities [21]. Another type of light used in FCM for cell sorting is fluorescent light. It is emitted by fluorophores that have been stained with a specific structure on the cell.

In FCM, cells need to be labelled with fluorescent reagents which include a wide range of commercially available dyes, stains, monoclonal antibodies (mAbs) and quantum dots (QDs). A fluorochrome (fluorophore or simply flours) is a fluorescent chemical compound that absorbs and re-emits light of different wavelengths upon excitation. Fluorophores are typically directly coupled to antibodies to create labelled antibody regents (fluorophore-conjugated antibodies). There are a number of commonly available fluorochromes in the market, such as fluorescein isothiocyanate (FITC), peridin chlorophyll protein (PerCP), allophycocyanin (APC) and phycoerythrin (PE) [10]. There are other specific dyes for cell functional studies, such as DNA dyes (e.g., ethidium bromide [EtBr], propidium iodide [PI]), glutathione-sensitive dyes (e.g., 7-amino-4-chloromethylcoumarin, monochlorobimane), calcium-sensitive dyes (e.g., fluo-4, fura red) and hydrogen peroxide (H_2O_2)–responsive dyes (e.g., dihydrorhodamine 123, 2′,7′-dichlorofluorescein) [10,22–24]. In addition, quantum dots (QDs), a new class of inorganic fluorochromes produced from semiconductor materials, is now emerging in polychromatic flow cytometry [25].

3. Current applications of FCM in immunological studies of molluscs

3.1. Total haemocyte counting

As haemocytes are chief immuno-effector cells that play a critical role in the innate immune system of molluscs, alternations of total haemocyte counts (THC) in haemolymph are an important immunological parameter to assess the health state of the host. In fact, changes in circulating haemocytes represent a common response of molluscs to infections or diseases [26–29] and environmental stresses [30–32]. Traditionally, cell counting was carried out by microscopic methods [12,33,34] which are time-consuming and relatively less accurate. In this regard, FCM provides a fast, easy, convenient and affordable alternative for counting mollusc haemocytes. In principle, various DNA-binding dyes (e.g., SYTO 9, SYTO 13 and SYBR Green) are used to stain all cells with a nucleus (nucleated cells) which can be discriminated with debris and non-nucleated cells.

FCM has been intensively used to quantify circulating haemocytes of molluscs under normal conditions [35,36] and in response to environmental stress, such as bacterial infections [13,29,37,38] and temperature variations [39]. For example, THC variations of *Mytilus galloprovincialis* mussels have been reported when injected with living or heat-killed *Vibrio anguillarum* and *Micrococcus lysodeikticus* [13]. Nguyen et al. [29] observed a significant increase in THC in *Perna canaliculus* mussels at 6 h post-infection with *Vibrio* sp. (8.01×10^6 cfu $\cdot$mL^{-1}) compared to controlled mussels (2.68×10^6 cfu $\cdot$mL^{-1}).

THC is normally performed along with other parameters in multiparameter assays, such as viability [37], ROS [40] and apoptosis [40]. Some studies have used fixed haemocytes [35,36,39] and others have used fresh cells [41–43].

3.2. Cell types

Different types of mollusc haemocytes have different functions [44]. Hence, identification of cell subpopulations is important to understand the immune function of each cell type. In specialized flow cytometers with sorting capabilities, cell populations can be separated into subpopulations typically based on size, morphology and expression of surface proteins [11]. In immunological studies of molluscs, identification of haemocyte subtypes have been mostly based on relative flow-cytometric morphological parameters: FSC and SSC. These studies have led to the characterization of different haemocyte subpopulations of different molluscan species such as disk abalone (*Haliotis discus discus)*, spiny top shells (*Turbo cornutus*) [36], Eastern oysters (*Crassostrea virginica*) [45], Mediterranean mussels (*M. galloprovincialis*) [38] and European flat oysters (*Ostrea edulis*) [46]. For example, there are two types of haemocytes (blast-like cells and hyalinocytes) in *H. discus discus* and four main haemocyte types (blast-like cells, type I and II hyalinocytes and granulocytes) in *T. cornutus* [36]. Interestingly, differences in immune-related activities were observed among the cell types [36]. *C. virginica* haemocytes were grouped into three morphologically different subpopulations, including hyalinocytes, granulocytes and intermediate cells [45]. Similarly, haemocytes of *O. edulis* [46] and *M. galloprovincialis* [38] were classified into three subclasses, including small hyalinocytes, large hyalinocytes and granulocytes. Characterization of molluscan haemocyte subpopulations is often combined with other multiple parameter assays to determine differences between cell types in immune-related activities, such as phagocytosis [35,47], oxidative stress [48–50] and apoptosis [46].

3.3. Viability

A cell viability assay is the quantification of the amount of live or death cells in a population. The cell viability or cell death is an important parameter for health assessment of the host. In addition, it is often necessary to detect and exclude dead cells which could unwantedly uptake of fluorescent probes, resulting in artefacts. The Trypan blue staining technique is a routine method of viability determination [11,33]. In principle, live cells have intact cell membranes that exclude certain dyes (e.g., trypan blue, eosin, or propidium) whereas dead cells with damaged and permeable membranes do not. Stained cells are visually exampled with a hemocytometer under a conventional microscope to determine whether cells take up (blue cytoplasm) or exclude dyes (clear cytoplasm) [33]. However, this technique is time consuming and has many other limitations [33]. Alternatively, FCM is a rapid and reliable method which allows the analysis of not only cell viability, but a variety of parameters at the same time. In FCM, cells are stained with intercalating dyes that bind to DNA, such as PI, EtBr, 7-aminoactinomycin D (7-AAD). Hence, non-viable cells (dead cells) are characterized with high fluorescence whereas viable cells are non-fluorescent [11].

The viability is often assessed via viability assays which have been extensively applied to measure the viability of molluscan haemocytes in response to different stress conditions, such as pathogen infections or diseases [37], water contaminants [40], thermal stress [51] or other stress conditions [13,41,42]. The assay provides a quantitative number/percentage of live and dead cells in the population (Fig. 2). For example, decreases of haemocyte viability were reported in *P. canaliculus* exposed to *Vibrio* sp. [37] and copper [40]. Similarly, significantly slower percentages of live haemocytes were found in *C. virginica* oysters after the temperature increase [51].

The viability determination of mollusc cells is often combined with other parameters in multiple cellular parameter assays, using fluorescent dyes with very different absorptions and wavelengths. For instance, live/dead cell distinction with 7-AAD was used in combination with Annexin V apoptosis measurements to determine percentage of late apoptosis in *P. canaliculus* haemocytes *in vivo* challenged with *Vibrio* sp [52] or *Haliotis iris* haemocytes under different probiotic-supplemented diets [43]. Similarly, 7-AAD was combined with dyes for caspase-3/7 and mitochondrial potential to identify early and late apoptosis in *P. canaliculus* haemocytes *in vitro* exposed to copper [40].

Alternatively, cell viability can be assessed via light-scattering measurement without any staining. Dead cells or cells with damaged and permeable membranes possess a reduction of FSC signals and an increase in SSC signals. Hence, the combination of FSC and SSC parameters could be used to distinguish live cells and dead cells [11].

3.4. Phagocytosis

The internal defences of molluscs rely only on the innate immune system, and phagocytosis is the most important line of defense in this system [53]. Phagocytosis is the engulfment of foreign particles (bacteria, algae, cellular debris and protozoan parasites) by phagocytes. Granulocytes are phagocytic cells, and they play a prominent role in phagocytosis of molluscs while agranulocytes are less important for phagocytosis [44,47].

FCM provides unique integration of functional and phenotypic information for phagocytosis studies [11]. FCM has been used to identify the phagocytotic activity in haemocytes of molluscs under different stress conditions, such as salinity [54], temperature [54], heavy metals [14] and different contaminants [55]. In phagocytosis assays, molluscan haemocytes are often incubated with fluorescent target particles, such as fluorescent beads [14,35,36,46] or zymozan particles [18,56]. Phagocytosis is then measured as phagocytosis index which is determined by the percentage of phagocytic cells that had ingested beads. However, determination of phagocytic cells is different from study to study. Some authors defined phagocytic cells with at least one fluorescent bead [14,36], while others used at least two [57] or three fluorescent beads [51,58]. This makes it difficult to compare phagocytic activity levels across studies. Along with the phagocytosis index, Donaghy et al. [36] also used the mean number of beads per phagocytic haemocytes to identify phagocytic activities in haemocytes from *H. discus discus* and *T. cornutus* stimulated by latex beads. Although the phagocytosis index was similar between these two species, the mean number of engulfed beads was slower in *T. cornutus* than in *H. discus discus*.

Many authors compared phagocytotic activity between different cell types [35,47]. For example, Donaghy et al. [35] used FCM to identify different subpopulations of *Crassostrea ariakensis* haemocytes and phagocytic activities of each cell type. The results showed that granulocytes were the main phagocytic cells, while hyalinocytes also showed a certain level of phagocytosis, and no phagocytic activity was observed in the blast-like cells. Similarly, Aladaileh et al. [47] observed the ingestion of yeast cells in both granulocytes and hyalinocytes of Sydney rock oysters (*Saccostrea glomerata*), but granulocytes were more efficient phagocytes than hyalinocytes.

Upon phagocytosis of pathogen, phagocytic cells release ROS which triggers apoptosis in molluscan haemocytes [59,60]. On the other hand, phagocytosis of apoptotic cells helps to clean of unwanted cells or cell components that might trigger inflammatory response [61]. Hence, assessment of phagocytosis via FCM is often combined with simultaneous measurement of other functional parameters, typically oxidative stress [35,36,51,62,63] or apoptosis [63–65].

3.5. Oxidative stress

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO•) are naturally produced as by-products of normal metabolism of oxygen and in responses to endogenous and exogenous stimuli [66]. ROS generation plays an important role in different biological processes, cell signalling, homeostasis and protective mechanisms [60]. However, excess ROS production during endogenous and exogenous stimuli leads to an imbalance between ROS and antioxidants in favour of ROS which is called oxidative stress [66]. The excessive ROS production can cause oxidative damage to cells and tissues via degradation of DNA, proteins and lipids [60].

Originally, ROS production of molluscs was measured using reduction of nitroblue tetrazolium (NBT) assays [67–69] and luminol-

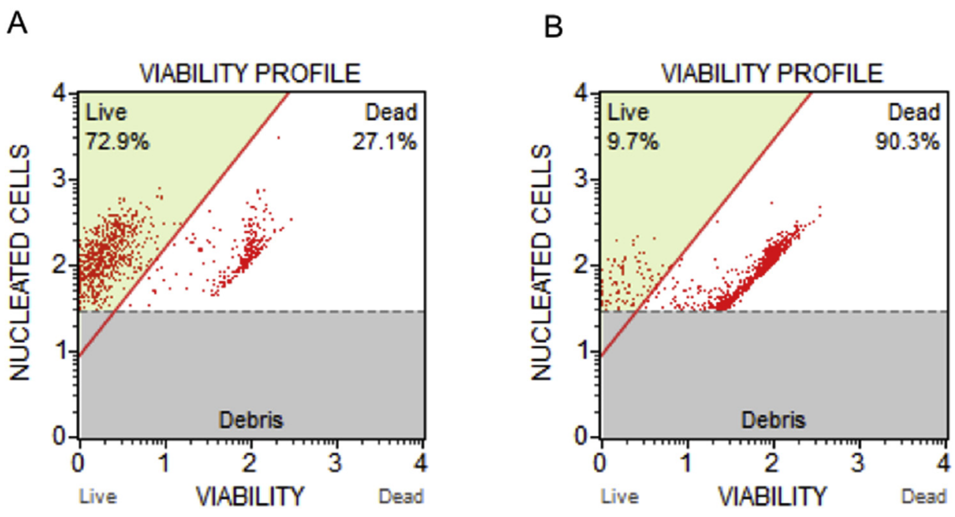

Fig. 2. Viability profile of *P. canaliculus* haemocytes under normal condition (control) (A) and *Vibrio* sp. infection (B). Viability of haemocytes was assessed by viability assay using a Muse™ Cell Analyzer. The red lines were used to separate live cells (left) and dead cells (right). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

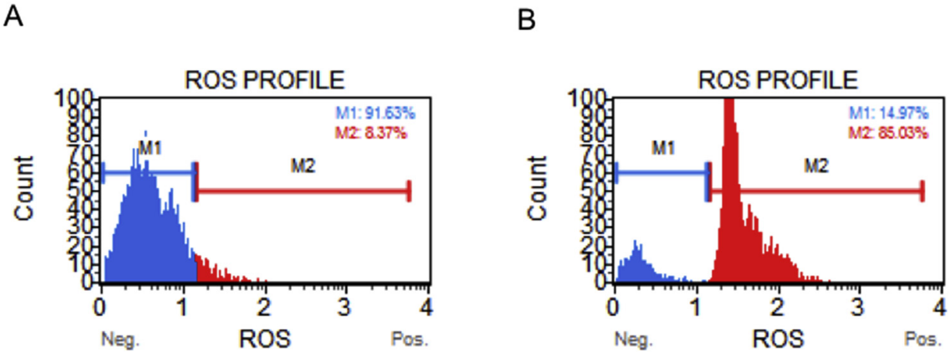

Fig. 3. ROS profile of *P. canaliculus* haemocytes under normal condition (control) (A) and *Vibrio* sp. infection (B). ROS percentage was determined by oxidative stress assay with DHE dye using a Muse™ Cell Analyzer. M1 indicates cell population without ROS (ROS_) while M2 is cell population with ROS (ROS+).

dependent chemiluminescence [70,71]. With its advantages, FCM has progressively replaced these assays for identifying ROS production. FCM assays use different dyes based on auto-oxidation, photochemical reactions, mitochondrial respiration and various enzymes (e.g., cytochrome P450, NADPH oxidase) [11]. Most of these dyes are photostable fluorogenic probes which are permeable DNA-specific dyes [11]. 2′,7′-dichlorofluorescein (H2DCF) and dihydroethidium (DHE), have been used extensively to measure H_2O_2 and O_2-, respectively, in mollusc haemocytes. DCFH-DA is a nonfluorescent fluorescein analogue which is a membrane permeable probe. The method used in molluscs studies was adapted from Bass et al. [72]. When added into haemocytes, DCFH-DA diffuses into the cell membrane where it is hydrolysed to 2′,7′-dichlorofluorescein (DCFH) by esterase enzymes. DCFH is then oxidized to DCF molecule by ROS (mostly of hydrogen peroxide and related superoxide species). DCF can be detected on the FL1 detector of the flow cytometer which results in green fluorescence, indicating haemocytes with oxidative activity.

DCFH-DA has been successfully used to determine the levels of H_2O_2 in mollusc haemocytes under normal conditions [50] or in response to pesticides [73], zymosan [74,75], heavy metals [76], temperature [77], pathogens [49,56], interactive effects of metals and pathogenic organisms [78] and other physiological conditions [79]. In addition, the DCF green fluorescence levels were used to evaluate ROS production of haemocyte subpopulations distinguished according to their relative size (FSC) and complexity (SSC) [48–50]. For instant, during the infection with *Vibrio aestuarianus* strain on *C. gigas*, Labreuche et al. [49] found a strong enhancement of ROS production in hyalinocytes and granulocytes which was higher in granulocytes than in hyalinocytes. In contrast, very low ROS production was observed in small agranulocytes. Similarly, Wang et al. [50] found higher ROS production in granulocytes than in hyalinocytes of *Perna viridis* mussels.

Another common dye used in detection of superoxide production in mollusc studies is dihydroethidium (DHE). DHE is a cell permeable dye which freely permeate cell membranes and react with superoxide anions to form 2-hydroxyethidium which intercalates with DNA resulting in red fluorescence [80–82]. This dye has been used to measure ROS production in mollusc haemocytes under different external stimuli, such as pathogen infections [37,52], LPS [41], heavy metal [40,83], pesticide [73] and storage conditions [42]. As an example, Nguyen et al. [37] used Muse® Oxidative Stress kit (EMD Millipore) based on DHE staining to measure ROS production in *P.canaliculus* haemocytes challenged with *Vibrio* sp. This assay requires no wash and takes 30 min to incubate 20 μL of haemocytes (1×10^6 cell·mL^{-1}) in 180 μL of working solution containing DHE dye at 37 °C. The measurement was performed via Muse® Cell Analyzer which normally takes less than 1 min per sample, depending on cell concentration and desired number of events to acquire. The assays provide total cell count and percentage of cells produced ROS in the population (Fig. 3).

3.6. Apoptosis

Apoptosis is a form of programmed cell death that occurs in multicellular organisms during various important cellular processes, ranging from embryonic development to host defense against pathogen infections [84]. Apoptosis is generally characterized by distinct morphological and biochemically characteristics [84] which are regulated via two major pathways: intrinsic and extrinsic pathways [59,85–88]. The intrinsic pathway (mitochondrial-mediated apoptotic pathway) is stimulated by various types of intracellular stressors, while the extrinsic pathway (death receptor-mediated apoptotic pathway) is activated by external stimuli. Apoptosis is a conserved mechanism across taxa and components of apoptotic pathways in molluscs seem to be similar to those of vertebrates with some unique features [89,90]. Apoptosis is an important internal defence mechanisms in molluscs in response to environmental changes, pollutants and pathogens [89].

FCM has become the most widely used method of choice for multiparametric analysis of apoptosis [91]. Many distinct characteristics of an apoptotic cell can be measured using FCM, including plasma membrane changes, changes in mitochondrial transmembrane potential, caspase activation and DNA cleavage [10,92]. In immunological studies of molluscs, annexin V, mitochondrial transmembrane potential (Δψm), capsase activation are common biomarkers used in characterization of apoptosis.

Annexin V is a cellular protein in the annexin group that has the ability to bind to phosphatidylserine (PS) of cells. Translocation of PS from the cytosolic side of the intact plasma membrane to the extracellular surface is a hallmark of early apoptosis [91]. The Annexin V assays utilize Annexin V as a useful marker for detection of PS on the external membrane of apoptotic cells during early apoptosis [93]. Annexin V staining must always be used in conjunction with a cell dead marker (e.g., PI, 7-AAD) as an indicator of the integrity of the cell membrane to rule out “leaky” necrotic cells as well as distinguish between live, dead and apoptotic cells [11,91]. Annexin V assays have been successfully used to characterize early apoptotic haemocytes of molluscs under different experiments, such as responses of *P. canaliculus* to *Vibrio* sp. infection [52], *Haliotis iris* to multi-strain probiotics [43], and cadmium-induced apoptosis in oyster haemocytes [94]. As an example, Nguyen et al. [52] used a Muse® Annexin V and Dead Cell assay with Annexin V for apoptosis and 7-AAD for dead cells to characterize the apoptosis in *P. canaliculus* following *Vibrio* sp. infection. They observed the increase of apoptotic cells and dead cells along with the decrease of live cells upon *Vibrio* sp. infection (Fig. 4).

3.6.1. Mitochondrial transmembrane potential

During the early apoptotic process, cells decrease mitochondrial transmembrane potential (Δψm) before rupture of the plasma membrane [95,96]. Thus, Δψm loss is another reliable indicator of apoptosis. Assessment of Δψm can be performed using fluorescent dyes, such as JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′- tetraethylbenzimi-dazolylcarbocyanine iodide), 3,3’ dihexyloxacarbocyanine iodide (DIOC6), JC-10, tetramethylrhodamine methyl ester perchlorate (TMRM) [10]. This method is based on the fact that these dyes are cationic probes that accumulate in healthy mitochondria resulting in high fluorescence. In cells with depolarized mitochondria, the dye is released, thereby resulting in a decrease in fluorescence and a downward shift. These dyes are often used with PI or 7-AAD to distinguish between apoptotic and nonapoptotic cells.

Determination of Δψm has become a method of choice for characterization of apoptosis in molluscs [40,46,97,98]. JC-10 is a common dye which has been used to measure the Δψm in *O. edulis* haemocytes exposed to UV [97] and the parasite *Bonamia ostreae* [99], and *C. gigas* haemocytes through the use of chemical inhibitors [98]. Others used tetramethylrhodamine, ethyl ester perchlorate (TMRE) for monitoring changes in Δψm in *M. galloprovincialis* haemocytes exposed to commercial nanosized carbon black (NCB) [100] and nanoparticles [101,102]. Nguyen et al. [40] used a commercial MitoPotential dye and 7-AAD to measure the Δψm and cell death in *P. canaliculus* haemocytes exposed to increasing concentrations of copper (Fig. 5). Furthermore, the use of DIOC6 dye for measurement of Δψm combined with morphometric parameters (FSC and SSC) were applied to compare the Δψm in different sub-populations of *O. edulis* haemocytes [46].

3.6.2. Caspase activation

Caspase activation is another hallmark of apoptosis [92]. Caspases (cysteinyl-directed aspartate-specific proteases) constitute a family of protease enzymes which are the key molecular components of both intrinsic and extrinsic pathways [103,104]. Functionally, caspases are categorized into three major types, including initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase- 3,-6,-7) and inflammatory caspases (caspase-1,-4,-5,-11,-12,-13) [105,106]. Several caspases have been identified in different mollusc species, including caspase-2 [88,90,107], caspase-8 [90,108,109] and caspase-3/7 [90,110].

A number of assays have been developed for detection of apoptosis based on the pivotal and early involvement of caspases in cell death events [11,111]. Activation of caspases can be identified by different techniques, such as immunoblotting, flow cytometry and microscopic techniques [111]. In FCM, cell populations are stained with the intracellular mAbs or small inhibitor peptides conjugated to a fluorophore which are assigned as affinity ligands to active site of relevant caspases [11]. Hence, antibodies/inhibitors-caspase complexes are detected based on the presence of the fluorescent tag (FITC or SR) inside viable cells [112,113]. Caspase dyes are often used together with dead cell stains (e.g., 7-AAD, PI) to distinguish between apoptotic, live and necrotic cells.

Caspase-3 is a frequently activated death protease in apoptotic cells of both vertebrates [114] and invertebrates [94,110,115,116]. Using the FCM method, increases of caspase-3 activation have been reported in *P. canaliculus* haemocytes exposed to copper [40] or LPS [41], and *Mytilus edulis* haemocytes exposed to copper [117]. As an example, Nguyen et al. [40] used the Muse™ Caspase-3/7 kit to quantitative measurements of apoptotic status in *P. canaliculus* haemocytes *in vitro* exposed to copper. The kit utilizes a Muse™ Caspase-3/7 reagent

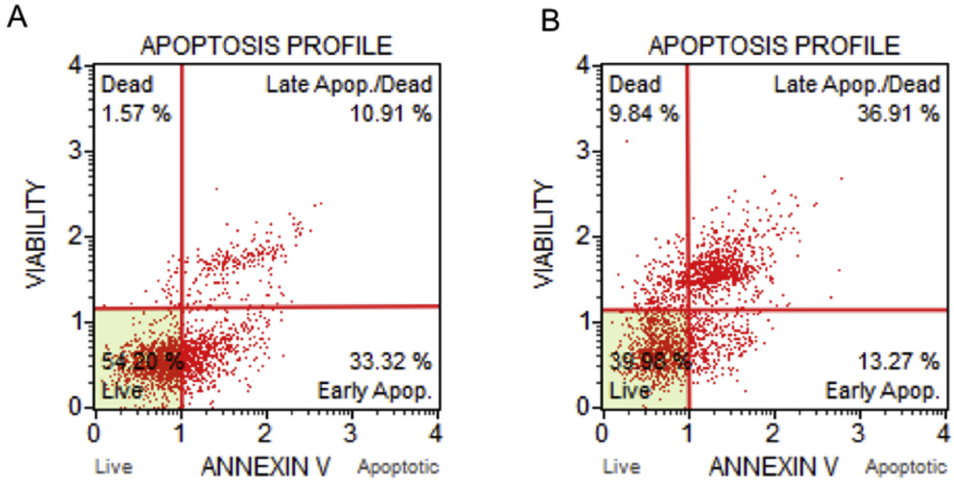

Fig. 4. Apoptosis profile of *P. canaliculus* haemocytes under normal conditions (A) and *Vibrio* sp. infection (B). Detection of apoptosis by concurrent staining with Annexin V and 7-AAD. Bivariate analysis of Annexin V/7-AAD staining distinguishes four populations of cells, including non-apoptotic cells (Annexin V^-, 7-AAD^-), early-stage apoptotic cells (Annexin V^+, 7-AAD^-), late-stage apoptotic cells (Annexin V^+, 7-AAD^+) and dead cells (Annexin V^-, 7-AAD^+) [52].

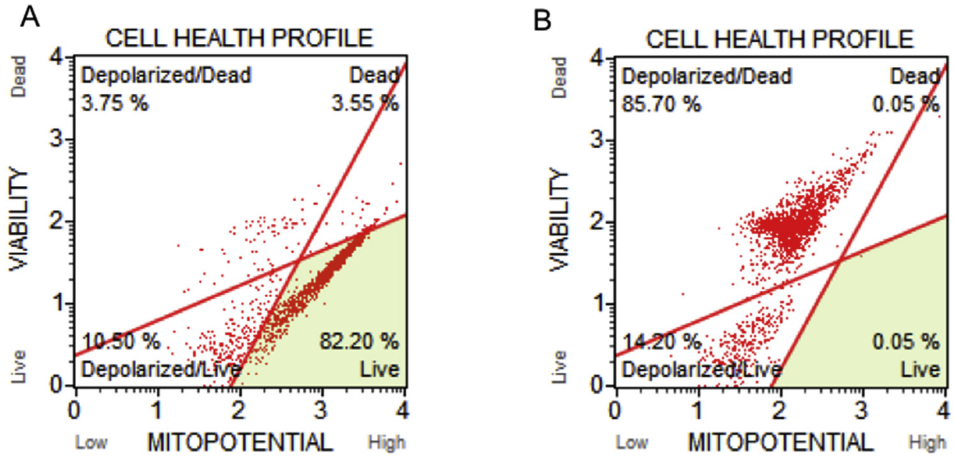

Fig. 5. Apoptosis profile of *P. canaliculus* haemocytes under normal conditions (A) and copper exposure (B). Detection of apoptosis was conducted by MitoPotential assay which provides four distinguishable cell populations, including live cells with depolarized mitochondrial membranes, live cells with intact mitochondrial membranes, dead cells with depolarized mitochondrial membranes and dead cells with intact mitochondrial membranes [40].

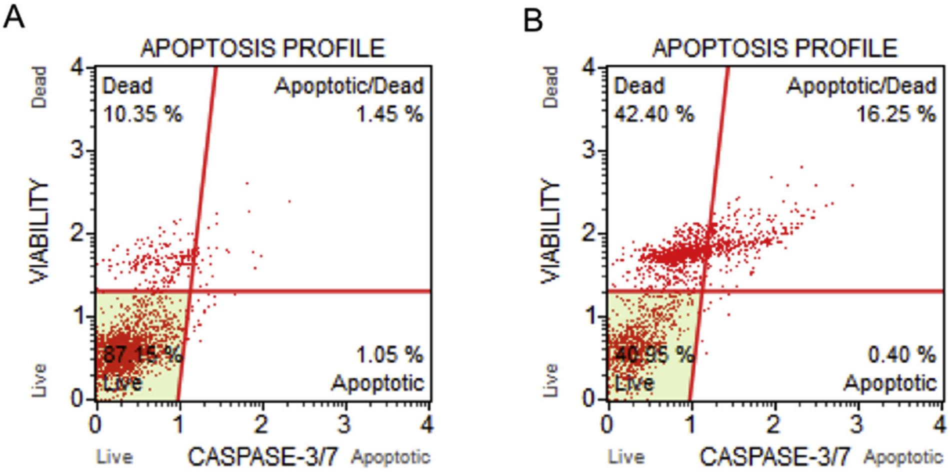

Fig. 6. Apoptosis profile of *P. canaliculus* haemocytes under normal conditions (A) and copper exposure (B). Apoptotic changes were detected using Muse™ Caspase-3/7 assay. The assay provides four distinguishable cell populations, including live cells, early apoptotic (live) cells, late apoptotic (dead) cells and dead cells.

NucView™ for the detection of caspase-3/7 activity along with 7-AAD as a dead cell marker. The assay provided relative percentages of four cell subpopulations, including live cells, early apoptotic cells, late apoptotic cells and dead cells (Fig. 6). They observed significant increases of dead cells and apoptotic cells in copper-exposed haemocytes compared to the control haemocytes.

3.7. DNA content and cell cycle characteristics of haemocytes

FCM is the most valuable technique of choice for cellular DNA content and cell cycle analysis [118]. It allows for the characterization of cells in the major phases of the cell cycle (G0/G1, S and G2/M), determining frequency of apoptotic cells based on fractional DNA content, and additionally detecting DNA amounts (polyploid or aneuploid) of the cell population [118,119]. Comparatively to other available traditional methods (e.g., histology and hemocytology), FCM is a rapid, accurate, non-subjective and cost-effective approach [118]. The analysis of DNA content and cell cycle characteristics are based on the staining of DNA with fluorescent dyes (e.g. PI, 7-AAD, Hoechst stains, TO-PRO-3, SYTOX, acridine orange, pyronin Y) [11].

FCM has been extensively used to characterize DNA content and cell cycle in various mollusc species [118,120–125]. For example, FCM has been successful used to determine the DNA content and genomic characteristics of abnormal cells affected with hemic neoplasia in the haemolymph of many molluscan species, such as in the *Mytilus* mussels [126], the soft shell clams (*Mya arenaria*) [121,127] and in the common cockles (*Cerastoderma edule*) [122,125]. These studies have successfully distinguished between negative and heavily affected bivalves using FCM. Recently, Benabdelmouna and Ledu [118] used FCM to determine genomic abnormalities in haemocytes of blue mussels, *Mytilus* spp. associated with mortality outbreaks in from the Atlantic coast of France. Different thresholds of genomic abnormalities (GA%) based on the percentage of haemocytes in S-G2/M phase were set up at individual and populations levels. The results provide evidence of heavy genomic abnormalities and GA% was found to be significantly predictive of the final mortality. Together, these studies demonstrate that FCM is a powerful tool for accurate diagnosis of diseases in marine bivalves.

3.8. Protein analysis

FCM has been appears to be an important tool for future protein analysis which could be applied for routine assays to quantify the number of proteins expressed in a cell and on the cell surface [128]. This is based on the principle that intracellular proteins in the complex mixture can be specifically labelled using fluorophore-conjugated antibodies which can be quantify using FCM.

There are a few studies using FCM for protein analysis in mollusc species [16,129]. For example, Friedl et al. [16] measured the total protein in haemocytes of the American oysters (*C. virginica*) by FCM. They observed a broad uniform distribution of proteins which was similar to that obtained for cell size. Rey-Campos et al. [129] used FCM to measure myticin C, the most expressed antimicrobial peptides in mussels, in different haemocyte populations of *M. galloprovincialis* mussels infected with *Vibrio splendidus*, and observed the decrease in the number of myticin C positive haemocytes after infection.

4. Challenges and perspectives

One of the big challenges of working with molluscan haemocytes is cell aggregation. To prevent haemocytes from clumping during bleeding, many different types of anti-aggregant solutions have been used, such as Alsever's solution, modified Alsever's solution, heparin sodium solution and modified Leibovitz L15 [130–133]. However, the use of anticoagulant for molluscan haemocytes may inhibit haemocyte activities [134]. For example, the use of modified Alsever's solution was reported to decrease oxygen metabolite production in bivalves [135]. The aggregation of molluscan haemocytes could be reduced or prevented by the maintenance of haemocytes on ice or low temperature, such as 4 °C [136,137]. Hence, in our recent studies, we have simply used cold filtered artificial seawater to mix with haemolymph in order to prevent cell clotting [37,40–42,52]. This method requires a minimal sample manipulation (no lyse, no cell wash) to mimic physiological conditions.

In addition to anticoagulants, the use of staining dyes may be toxic for cells. It may cause a loss in cell viability and in certain situations even apoptosis or severe damage [11]. Hence, it is important to take into account the toxicity of the dyes for the haemocytes and the combination of different assays is sometimes necessary to identify the true value of the measured parameters.

Recently, there has been an emergence of metabolomics applications in molluscan immunity studies [40,41,138,139]. Many authors also combined FCM and untargeted GC-MS metabolomics in molluscan immunology [37,40,52]. For example, Nguyen et al. [40] employed FCM and GC-MS metabolomics to characterize effects of copper on haemocytes. Along with the increase of an oxidative stress biomarker, ROS, in copper-exposed haemocytes, they found the accumulation of glutathione and other metabolites in glutathione pathways, suggesting the role of glutathione as a metabolite maker of oxidative stress. Similarly, the increases of apoptosis hallmarks including caspase 3/7 activation and $\Delta\psi m$ loss were found to be linked with the increase in alanine and decrease of glutamic acid in the taurine metabolism, which plays a key role in apoptosis regulation [40]. Hence, these examples demonstrated that the combination of FCM and metabolomics could expand the number of cellular and molecular markers to provide an extensive evaluation of functional markers. Such kind of integrated approach could be expanded to combinations between multiple FCM parameters and other omics (e.g., transcriptomics, proteomics) or integrated omics to create a detailed picture of immune responses within cells and generate accurate biomarker signatures of molluscan diseases.

Recent advances in technology have led to a comprehensive range of innovative flow cytometers. Many of these are small in size and simple in operation, and allow for routine sampling in the field. As an example, the Muse® Cell Analyzer has been extensively used in our lab for both field and laboratory measurement of many cell health parameters of molluscs. At the moment, only the cell count and viability assays appear to be suitable for field sampling because they are simple and fast and the dye used can just be stored at cool temperature (2–8 °C). Other assays, such as oxidative stress and apoptosis, require the dyes to be stored at −20 °C and they need long incubation times (30 min), which are not likely to be suitable for the field. Hence, future modifications of storage conditions of these dyes or greater molecular and cellular labelling techniques will make other assays more applicable in the field. Furthermore, continued advances in instruments along with the decrease in the price will allow for the wider use of FCM in essentially any setting.

The use of FCM in immunological studies has advanced in vertebrates with a diverse range of measurable parameters [10,11]. However, FCM parameters used in molluscan immunological studies are limited to a few measurable parameters. This is a great opportunity for marine scientists to continue to explore the advantages of modern FCM to provide insights into molluscan immunology. However, the lack of standardized cellular reference materials for molluscan haemocytes remains a big challenge for the development and validation of new assays. Among the current parameters used, only viability and cell subpopulations has been validated [12,13,36,140]. There are currently no guidelines for the validation of FCM methods to be used in molluscan species. Hence, there is a need to conduct appropriate validations for the numerous FCM assays used in molluscan immunological studies.

5. Concluding remarks

A variety of FCM assays currently exist for immunological studies of molluscs which have enabled deep understanding of molluscan immunology. FCM analyses in molluscs have focused on characterization of cell types, response of haemocytes to a particular stress, diseases or toxic agent and diagnosis of diseases. However, applications of FCM in marine molluscs as well as in invertebrates are quite limited compared to those in vertebrates. To this end, marine scientists could take advances by applying and optimizing FCM methodologies that have been developed for mammalian cells for studies in mollusc species. As a tool, FCM can be used in combination with other techniques, such as omics approaches, which have advanced significantly in recent years. Ultimately, this will lead to a more improved understanding of molluscan immunology, and contribute to defining accurate biomarker signatures of diseases or stress conditions within wild and aquaculture settings.

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