

Review

## Flow cytometry as a tool for analysing invertebrate cells

A Cossarizza<sup>1</sup>, M Pinti<sup>1</sup>, L Troiano<sup>1</sup>, EL Cooper<sup>2</sup>

<sup>1</sup> Department of Biomedical Sciences, University of Modena and Reggio Emilia, Modena, Italy

<sup>2</sup> Department of Neurobiology, UCLA School of Medicine, Los Angeles, CA, USA

Accepted April 8, 2005

---

### Abstract

Flow cytometry (FCM) is a powerful tool that allows analysis of thousand of cells in a few seconds, at the single cell level. In the last 15 years, researchers have used FCM to investigate the cellular machinery of invertebrates. Analyses have focused on functions linked to innate immunity, such as phagocytosis and natural killer cell activity, as well as on the sensitivity of invertebrate cells to a particular stress or to a toxic agent. Further, FCM has been employed to recognize antigens, or at least immunodominant epitopes, shared in common with mammalian cells, including human leukocytes. In this review, main studies that have utilized FCM to investigate either phenotype and functions of invertebrate cells are reported and discussed.

**Keywords:** invertebrate; flow cytometry; immunology

---

### Introduction: diversity of approaches and principles of flow cytometry (FCM)

Recently, a consistent number of scientists working in different fields have utilized flow cytometry (FCM) for numerous analyses. FCM has indeed impacted both basic cell biology and clinical medicine in a significant manner, and instruments are present almost everywhere. According to the essential principle of FCM, single cells (or particles, or organelles) suspended within a stream of liquid pass through a light source (usually a laser beam tuned at 488 nm) focused on a minute region. During this passage, cells are interrogated individually thus providing relevant numbers of information. Basically, signals generated by cells passing through the laser beam are spectral bands of light in the visible spectrum, which represent the detection of various chemical or biological components, mostly fluorescence. Since flow cytometers can analyze single particles or single cells, it is possible to separate them into populations based upon differences in any of the variables that can be measured on each particle/cell.

Currently, the number of these variables is consistently high, and several instruments allow identification of more than a dozen different parameters in the same particle/cell. It is then possible to separate these populations electronically, and to identify them using multivariate analysis techniques.

FCM uses several fluorescent molecules that are attached by one means or another to the particle or cell of interest. The fluorescent probe might be membrane bound, cytoplasmic, or attached to nuclear material. To recognize specific receptors present on the plasma membrane, as well as to identify intracellular antigens or to calculate the amount of a given molecule within a cell, it is a common practice to use monoclonal or polyclonal antibodies directly conjugated to fluorescent dyes.

Particles of almost any nature can be evaluated by flow cytometry. They can be very small, even below the resolution limits of visible light, because they can be detected by their fluorescent signatures. Similarly, depending on the structure of the flow cell and fluidics, particles as large as several thousand microns can be evaluated.

FCM allows the evaluation of thousands of events in a very short time. For example, the most common instruments can detect hundreds of cells per second, measuring up to 5 parameters; sophisticated systems exist that can run particles at rates approaching 100,000 events per second while collecting 10 to 20 parameters from each particle. Finally, it is possible to separate single particles/cells physically from mixed

---

*Corresponding Author:*

Andrea Cossarizza  
Dept. Biomedical Sciences, University of Modena and Reggio Emilia, Via Campi 287, 41100 Modena, Italy.  
E-mail: cossarizza.andrea@unimore.it

populations and, by using the sorting option eventually present in the instrument, to place them into a defined location for cloning, or for further molecular analysis.

### **FCM analysis of cells from *Planorbarius corneus* using antisera to vertebrate bioactive peptides**

The first studies that employed FCM in invertebrates were devoted to the analysis of epitopes/molecules present in cells from the mollusc *P. corneus* that were able to cross-react with human molecules. The starting point was represented by immunohistochemical data, when the attention was devoted to the detection of immunoreactive molecules in round and spreading hemocytes of *P. corneus* (Ottaviani and Cossarizza, 1990). Antisera to vertebrate (mainly human) bioactive peptides were used, and it was found that spreading hemocytes had alpha 1-antichymotrypsin-bombesin-, calcitonin-, CCK-8 (INC)-, CCK-39-, gastrin-, glucagon-, Met-enkephalin-, neurotensin-, oxytocin-, somatostatin-, substance P-, VIP-, and vasopressin-immunoreactive molecules, while round hemocytes were only positive to anti-bombesin, anti-calcitonin, anti-CCK-8, anti-CCK-39, anti-neurotensin, anti-oxytocin, anti-substance P and anti-vasopressin antibodies. FCM, whose sensibility is clearly much higher than that of a microscope, was then used to confirm the presence of ACTH and  $\beta$ -endorphin immunoreactive molecules in the same invertebrate (Ottaviani *et al.*, 1990). In the hemolymph of these animals, two main types of cells exist, *i.e.* round (RH) and spreading hemocytes (SH) (Ottaviani, 1991). SH but not RH were positive to antisera recognizing vertebrate ACTH and  $\beta$ -endorphin. Interestingly, only by means of analyzing physical characteristics (dimension, granularity) of cells from invertebrate hemolymph, FCM allowed identification of the same cell types that had been described morphologically long before (Fig. 1). Interestingly, similar data on the presence of ACTH-immunoreactive molecules were obtained in another mollusc, such as *Lymnea stagnalis* (Ottaviani *et al.*, 1991), and in leukocytes from the frog *Rana esculenta* (Ottaviani *et al.*, 1992). The functional capacity of *P. corneus* spreading hemocytes, along with the presence of ACTH-like molecules (*i.e.*, reactive with antibodies directed against human ACTH) in other species, including *Carassius carassius var. auratus* and *Coris julius* (Pisces), *Hymenochirus gillii* (Amphibia), *Podarcis muralis* (Reptilia), and *Gallus domesticus* (Aves) suggested that cells with phagocytic activity, likely the oldest natural immune response, may represent a suitable model to unravel the tangled web of the common ancestor of the immune and the neuroendocrine systems.

### **Evidence of cytotoxicity in molluscs**

The investigation on natural immunity in *P. corneus* continued by evaluating the capacity of its cells to kill targets in a non-MHC restricted manner (Franceschi *et al.*, 1991). Natural killer (NK) cells represent an important form of cell recognition that results in cytotoxicity, and it was predicted that NK-like activity had to be preserved throughout phylogenetic

development (Cooper *et al.*, 2002). Nonadherent round hemocytes were able to lyse the K562 human target cell line in a short-term NK cytotoxicity test. This NK-like activity, severely reduced after 18 hour incubation at 24° C, was maintained by the presence of human recombinant interleukin-2 (IL-2). A further analysis performed by mouse anti-human monoclonal antibodies and cytofluorimetric analysis revealed that both SH and RH reacted with several antibodies, including those directed against epitopes typical of mammalian NK cells, and against different cell adhesion molecules (Fig. 2). These data suggested the hypothesis that a primitive NK-like activity appeared early in evolution, and is not shared by phagocytic cells.

### **Possible role of interleukins**

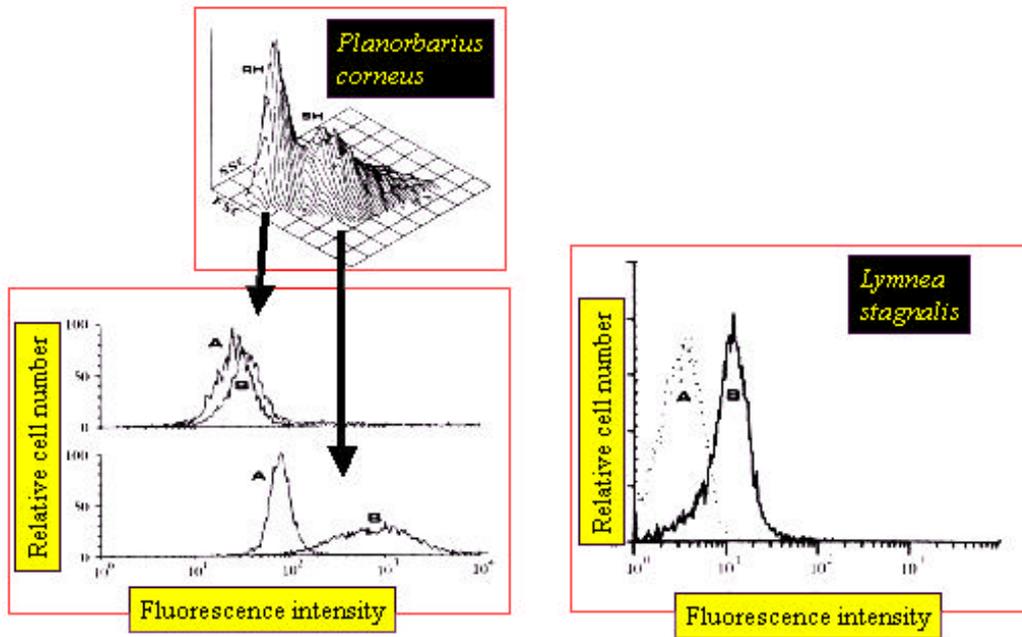
A support for this hypothesis came from the cytofluorimetric observation that IL-2 could compete with corticotropin releasing factor (CRF) by binding to the receptor that is able to bind human IL-2, present on the plasma membrane of molluscan hemocytes (Fig. 3). At the functional level, pre-incubation of hemocytes with IL-2 or anti-IL-2 monoclonal antibody (mAb) significantly reduced or completely eliminated the CRF-induced release of biogenic amines. These findings are compatible with the presence of a unique (ancestral?) receptor on molluscan hemocytes, capable of binding both CRF and IL-2, two key molecules of the neuroendocrine and immune system (Ottaviani *et al.*, 1994).

Subsequently, using FCM it was observed that spread cells present in the hemolymph of the mussel *Mytilus galloprovincialis* expressed three IL-2 receptor (IL-2R) subunits:  $\alpha$ ,  $\beta$  and  $\gamma$ . Mussel IL-2R $\alpha$  and IL-2R $\beta$  subunits displayed a molecular weight similar to those in vertebrates tissues, whereas mussel IL-2R $\gamma$  was smaller than that in vertebrates. Both lipopolysaccharide (LPS) and IL-2 induced IL-2R $\alpha$  expression, and such induction depended on the concentration of both agonists (Barcia *et al.*, 1999). Stimulation with LPS, IL-2 or platelet-derived growth factor also resulted in an increased release of dopamine, adrenaline and noradrenaline in the culture medium (Cao *et al.*, 2004).

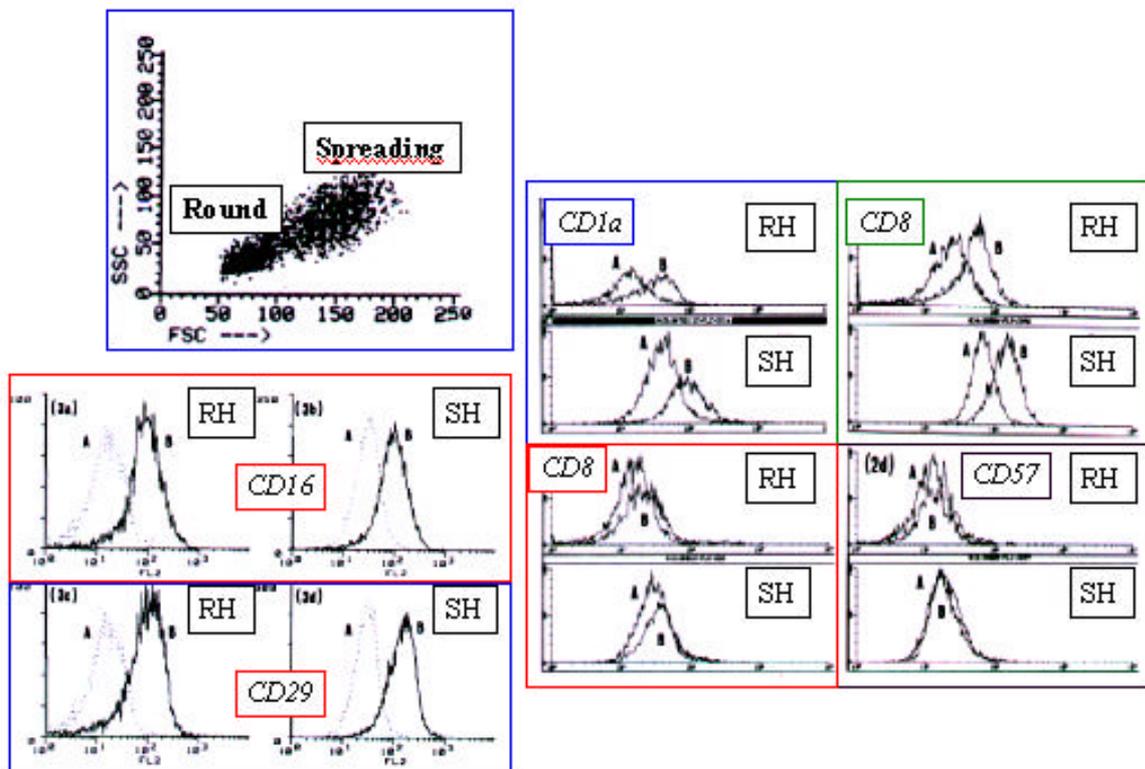
### **Identification of cell shape and surface antigens in other species, and relevance to tumor cells and environmental chemicals**

FCM proved to be a powerful technique for the analysis of the heterogeneity of cell populations in a variety of invertebrates. Studies were performed on hemocytes derived from individual *L. stagnalis*, and revealed that cell sizes were comparable in all 40 specimens studied, and was not affected by age or by infection with *Trichobilharzia ocellata* (Amen *et al.*, 1992).

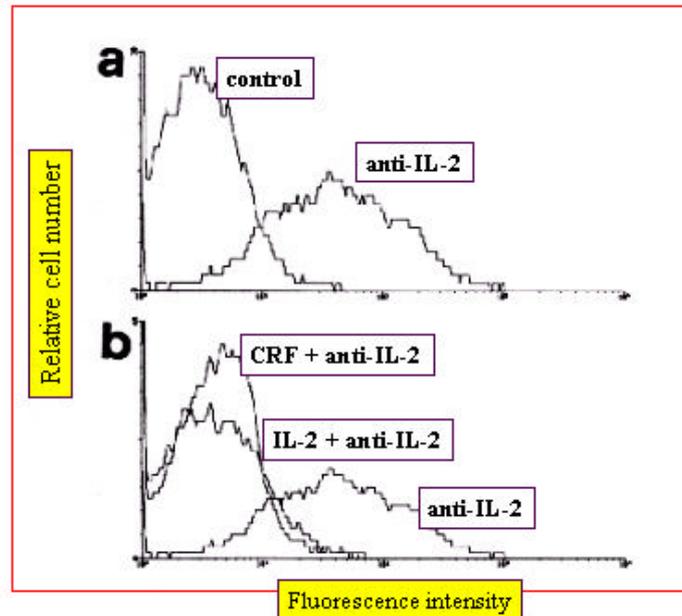
A monoclonal antibody of the clam was generated to normal hemocytes of *Mya arenaria* (White *et al.*, 1993). The antibody, designated 2A4, was evaluated by ELISA, immunocytochemistry, Western blotting, and finally FCM. *M. arenaria* is a very interesting model, because it develops a leukemia



**Fig. 1** Presence of immunoreactive ACTH molecules on the cell surface of Spreading (SH) and Round (RH) hemocytes from *Planorbarius corneus* and from *Lymnea stagnalis*. A= control, B= stained cells. Modified from Ottaviani *et al.*, 1991.



**Fig. 2** Expression of epitopes recognized by mouse anti-human monoclonal antibodies in round (RH) or spreading (SH) hemocytes from *Planorbarius corneus*. A= control, B= stained cells. Modified from Franceschi *et al.*, 1991.



**Fig. 3** Expression of IL-2 like molecules on the plasma membrane of *Planorbarius corneus* revealed by anti-IL-2 mAbs, and inhibition of the staining with exogenous IL-2 or CRF. Modified from Ottaviani *et al.*, 1994.

detected first in the hemolymph and, as the disease progresses, in solid tissue. The 2A4 antigen was detected on 87 % normal adherent cells, but was lost as nonadherent leukemia cells proliferated. The mature leukemia cell neither expresses 2A4 nor can 2A4 be detected in the leukemia cell lysate. Since 2A4 reacts with a 130-kDa protein, it was suggested that p130 may be involved in the regulation of cell adhesion.

Subsequently, the same investigators evaluated the reactivity of both normal and tumor cells from *M. Arenaria* to a polyclonal antibody to polychlorinated biphenyls (PCBs) (Harper *et al.*, 1994). FCM was indeed used to ascertain both leukemia prevalence and PCB reactivity. Analytical chemistry was used to quantitate the amount of Aroclor 1254 (a widely studied PCB), per tumor cell population and compared directly to FCM results. The prevalence of leukemia consistently exceeded 60 % when clams were retrieved from New Bedford Harbor, a site heavily contaminated with PCBs. Both normal circulating cells and tumor cells were extremely reactive with the PCB antibody. When clams from two other sites were compared with clams from New Bedford Harbour, both disease prevalence and cell reactivity to the PCB antibody were significantly reduced. This study was the first that demonstrated the presence of PCBs in cell populations of marine invertebrates, and showed that the presence of PCBs *in vivo* was directly correlated with environmentally linked leukemia.

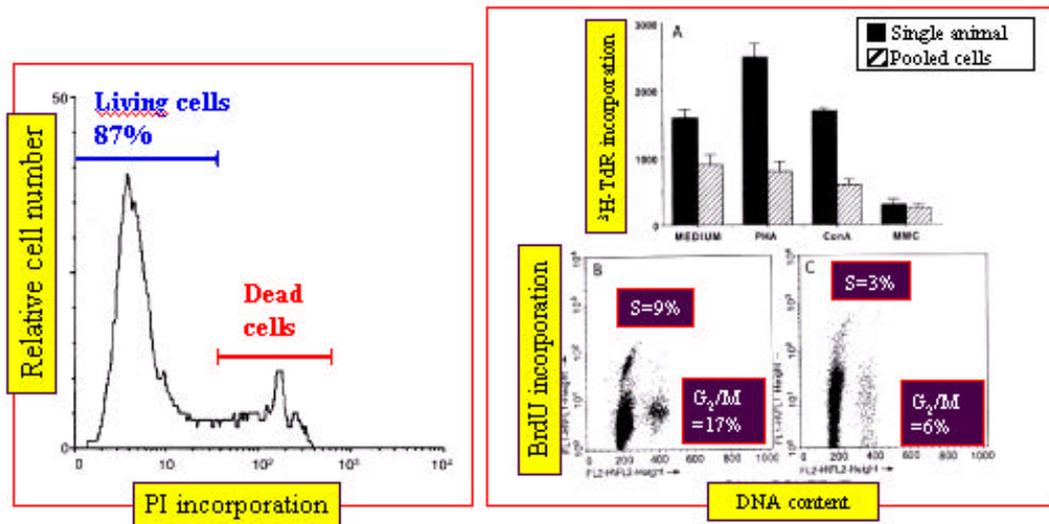
Exposure to ubiquitous environmental chemicals, such as polycyclic aromatic hydrocarbons, may affect the earthworm cellular immune-defense system. After exposure to soil contaminated with 7,12-dimethylbenzanthracene (DMBA), cellular functions of coelomocytes from the earthworm *Eisenia fetida* were examined by flow cytometry, that allowed to classify them as small and large cells (Komiyama *et al.*, 2004). Sexually mature animals were kept in dark at 18° C

with various doses of DMBA contaminated artificial soil for 7 days. Coelomocytes were harvested from earthworms and processed for *in vitro* phagocytosis and H<sub>2</sub>O<sub>2</sub> activity. Phagocytosis was assessed by ingestion of fluorescence beads and H<sub>2</sub>O<sub>2</sub> activity examined using 2',7'-dichloro-fluorescein diacetate. Cell functions were down regulated in a dose dependent manner after exposure to sublethal doses of DMBA. This study demonstrates the utility of flow cytometry to evaluate the biological activity of earthworm cells, and the effect of soil contamination for ecotoxicological studies.

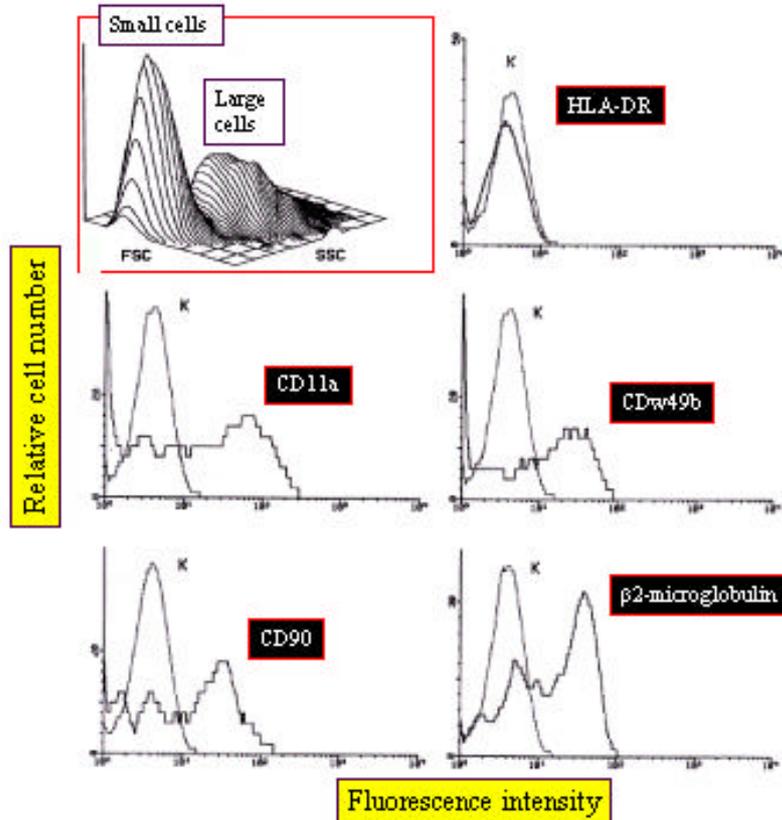
Age-related changes in the immunocyte population in *M. galloprovincialis* have been reported (Ottaviani *et al.*, 1998). In this mollusc, a young and an old stage belonging to the same cell type have been described. Interestingly, either in young or adult animals these cells were positive to the staining with mouse anti-human mAbs anti-CD5, -CD11b and CD16. Other molecules, such as CD10, a surface antigen known to be identical to neutral endopeptidase-24.11 (NEP), have been recently found in immunocytes of *M. galloprovincialis* (Caselgrandi *et al.*, 2000). Computer-assisted microscopic image analysis revealed NEP functional activity, *i.e.* the capacity to deactivate the PDGF-AB- and TGF- $\beta$ 1-induced changes in immunocyte shape.

#### **Cytotoxicity in earthworms: the model of *Eisenia fetida***

A cytometric approach has been used to investigate the main features of natural cytotoxicity in coelomocytes from *E. fetida*, that were used as effector cells against the human tumor target cell line K562 (Cooper *et al.*, 1995). To first assess the viability of effectors, DNA synthesis was tested and was higher in autogeneic (cells from one animal) than in



**Fig. 4** Allogeneic inhibition of cell proliferation. Left panel: Cell viability in freshly collected coelomocytes from *Eisenia fetida*. Right panels: A, proliferation of coelomocytes from single animals or pooled from different animals after stimulation with phytohemagglutinin (PHA), Concanavalin A (ConA) or inhibition with Mitomycin-C (MMC). Note that pooled cells showed an allogeneic inhibition. B and C: cytofluorimetric analysis shows the comparison between unstimulated cells from a single animal (B) or cells collected and pooled from different animals (C). Note the difference in the percentages of cells in the S or G<sub>2</sub>/M phases of the cell cycle. Modified from Coszarizza *et al.*, 1996.



**Fig. 5** Expression of epitopes recognized by mouse anti-human monoclonal antibodies in small and large cells from *Eisenia fetida*. k= control, unstained cells. Modified from Cooper *et al.*, 1995.

allogeneic (cells from two animals) coelomocytes. Cell cycle analysis revealed that autogeneic cultures showed significantly greater numbers of cells in S, G<sub>2</sub>, or M phases than allogeneic ones (Fig. 4). When autogeneic or allogeneic cells were kept together in culture, no significant cell killing occurred in either, while autogeneic but not allogeneic cultures could kill K562 target cells. Cytotoxicity was dependent upon membrane binding between small, electron-dense coelomocytes and targets; it was enhanced by adding the lectin phytohemagglutinin. The heat labile supernatant from autogeneic but not allogeneic cultures killed K562 targets. Recognition of, binding to, and killing of foreign cells in a natural killer cell-like reaction may reflect natural immunity in earthworms.

Subsequently, it was found that earthworm coelomocytes effect cytotoxicity at significantly high levels also against the NK-resistant target cells (such as the U937, BSM, and CEM cell lines) (Cossarizza *et al.*, 1996). Then, using FCM and mouse anti-human monoclonal antibodies, the two main types of cells present in *E. foetida* coelom were better characterized: small (8-11 µm), electron-dense cells (SC) were: CD11a+, CD45RA+, CD45R0+, CDw49b+, CD54+, HLA class II (DR)+,  $\alpha_2\text{-}\mu$ + and Thy-1+ (CD90+) (Fig. 5); large (12-15 µm), electron-lucent cells (LC) were negative for these markers. Both cell types were negative for other CD and MHC class I and class II markers. Subsequently, it was found that coelomocytes were positive to the staining with rat anti-mouse mAbs for CD90, CD5, CD8, CD45RA, CD45R0 and anti-perforin, negative for Ia, CD4 and CD11c (Komiya *et al.*, 2002). In general, only SC and to a lesser extent, LC reacted with these antibodies.

SC were active during recognition, rapidly binding to targets; LC were phagocytic (Cossarizza *et al.*, 1996). SC were able to exert rapid, significant, and equal levels of killing at 4, 20, and 37°C, suggesting that, as for phagocytosis, also primitive NK-like activity appeared early in evolution.

#### **FCM can be used to analyze cell organelles and different cellular functions**

The presence and functionality of mitochondria in terms of mass and membrane potential (MMP) have been investigated in both SC and LC from *E. foetida* at the single cell level by different FCM techniques (Cossarizza *et al.*, 1995). In comparison with SC, LC have a higher number of mitochondria, and, accordingly, showed a greater fluorescence intensity when mitochondrial mass was measured by nonyl acridine orange. To measure MMP, both the lipophilic cationic probe JC-1 and Rhodamine (Rh) 123 were used. Using JC-1, MMP was analyzed separately on SC and LC, and significant percentages of coelomocytes (> 95 % of SC and about 90 % of LC) displayed a high MMP. Adding 0.1 µM valinomycin (Val) caused most SC to depolarize, while this occurred in only a few LC. Rh123 gave different results: no effects of Val were observed either in SC or in LC. It was concluded that in coelomocytes there may be several energy-independent Rh123-binding

sites, and that it is possible to analyze mitochondrial parameters by FCM in intact invertebrate cells.

Earthworms possess also non-specific responses found in other complex metazoans. Coelomocytes from the *E. foetida* were further characterized by electron microscopy and FCM analyses, and the structural changes that occur when effector coelomocytes and target cells interact during cytotoxic activity were deeply investigated (Quaglino *et al.*, 1996). It was found that using *in vitro* cultures: 1) the two aforementioned earthworm cell types retained their morphological features; 2) their DNA content was significantly less than that of human lymphocytes and the erythromyeloid human tumor cell line K562; 3) significant percentages of coelomocytes were found to be in S or G<sub>2</sub>/M phases of the cell cycle. It is noteworthy that these two parameters were investigated by direct staining of DNA with fluorescent probes, as well as by the classical staining with bromodeoxyuridine (Dolbeare *et al.*, 1983). When cultivated alone, coelomocytes formed no aggregates. However, when mixed with K562 target cells, as described above, coelomocytes spontaneously killed tumor cells, and cytotoxic reactivity was accompanied by the formation of multiple aggregates similar to granulomas. These results are the first to describe this type of earthworm non-specific "inflammatory" response *in vitro* against xenogeneic tumor cells.

#### **FCM analysis of DNA content and Cell Cycle**

FCM has been used to quantify DNA content in invertebrate cells (Ulrich, 1990). Cell material from the diptera species *Chironomus thummi*, *Drosophila melanogaster*, *Calliphora vicina* and *Musca domestica* was analyzed using an impulse cytophotometer with a special quartz objective, that was especially manufactured for cytofluorometric investigations with the DNA-specific fluorochrome DAPI in combination with the protein fluorochrome sulforhodamine 101. The occurrence of heterogenous cell populations with aneuploid and polyploid DNA content within the cell material of different developmental stages of diptera species have been determined, whereby in larvae polyploid cell populations and in imagos aneuploid cell populations predominate.

More recently, studies were carried out to determine the alteration in DNA cell cycle characteristics of hemocytes from *M. galloprovincialis* collected at 17 different locations along the Adriatic coast, Croatia (Bihari *et al.*, 2003). FCM was used to connect possible genomic manifestation to urban and/or industrial waste, and the incidence of altered DNA profiles was investigated. Different alterations in cell cycle, mirroring either acute or cumulative genotoxic effects of the surrounding environment on mussel hemocyte DNA, were found. Among these, the most relevant were intraindividual genome size variability, aneuploidy and accidental apoptotic processes; normal cell cycle DNA profiles were obtained in 60.9 % individuals from all 17 sites. Molecular assays were used along with cytofluorimetric analysis of cell cycle, and confirmed the presence of DNA damages, such as alkali-labile sites and single-strand breaks, interstrand cross-links and DNA-protein cross-links (Bihari *et al.*, 2002).

A cell line from the insect *Spodoptera frugiperda* (SF9) was used to investigate the capacity of azadirachtin to alter the mitotic index (Salehzadeh *et al.*, 2003). FCM demonstrated that cells accumulated in the G<sub>2</sub>/M phase of the cell cycle, and that the effect was concentration-dependent. Azadirachtin had the same effect on C6/36 mosquito cells, but failed to affect L929 murine fibroblasts even at high concentrations. Experiments with colchicine and taxol showed similarities of action between azadirachtin and colchicine, and azadirachtin was apparently able to displace colchicine from specific binding-sites present in living insect cells.

*In vitro* analysis of the effects of 20-hydroxyecdysone (20E) on the cell cycle in IAL-PID2 cell line established from imaginal wing discs of *Plodia interpunctella* were reported (Mottier *et al.*, 2004). It was found that 20E induced an arrest of cells in G<sub>2</sub> phase, accompanied by a sharp decrease in the levels of cyclin A and B expression.

### Studies on DNA damages and apoptosis

Several groups have investigated by FCM either DNA damages or the induction of apoptosis in invertebrate cells. The terminal dUTP nick-end labeling technique (TUNEL) was used to detect *M. galloprovincialis* cells displaying DNA fragmentation within gill structures after treatment with tri-n-butyltin (TBT) (Micic *et al.*, 2001). DNA degradation as well as decreased number of cells in G<sub>0</sub>/G<sub>1</sub> were detectable after 1.5 hour of TBT incubation. Presence of apoptotic cells in mussels' gills was indicated by the selective loss of G<sub>2</sub>/M cells concomitant with the appearance of cells with decreased DNA content in the S and G<sub>0</sub>/G<sub>1</sub> regions of cell cycle. The effect of the TBT on cell cycle in a mussel gill was depending upon dose and exposure time.

Metabolites of salsolinol (SAL), an intraneuronal, dopamine-derived tetrahydroisoquinoline (TIQ), induce apoptosis in human dopaminergic neuroblastoma cells, and likely contribute to the killing of nigrostriatal dopaminergic neurons occurring in Parkinson's disease. Since insects employ dopamine and related catecholamines in a variety of processes including cuticular sclerotization and cellular immune reactions, the capacity of their cells to metabolize exogenous SAL was investigated (Ottaviani *et al.*, 2002). IPLB-LdFB cells from *Lymantria dispar* exhibited no significant increase in apoptosis when incubated for 48 hours with low concentrations of SAL (up to 1 mM). Apoptosis was observed only with the highest concentration of SAL tested (5 mM), but only 12.4 % of the cells manifested this form of cell death. The resistance of IPLB-LdFB cells to SAL-induced apoptosis was attributed to the ability of these insect cells to metabolize and/or detoxify the dopamine-derived catecholic TIQs.

The toxicity of the mycotoxins nivalenol (NIV), deoxynivalenol (DON), and fumonisin B1 (FB1) was studied in the lepidopteran *S. frugiperda* SF-9 cells by FCM (Fornelli *et al.*, 2004). NIV was significantly more toxic than DON, and both were significantly more toxic than FB1. Cell cycle distribution showed an arrest of cells in the G<sub>0</sub>/G<sub>1</sub> phase in the presence of all of the three compounds. Morphological evidence of

apoptosis was related to the toxicity of the substances in that the more toxic NIV induced late apoptosis, whereas DON and FB1 produced less-severe morphological changes characteristic of early apoptosis. It was concluded that NIV is more toxic than DON, which in turn is more toxic than FB1, and that these mycotoxins can modify the normal progression of the cell cycle and induce an apoptotic process.

### Assessing the response of invertebrate cells to fungi and bacteria

Innate immunity is strongly regulated by microbial products, and FCM can be added to the list of available methods to investigate this activity. Furthermore, FCM can provide a simple, reproducible, and sensitive method for evaluating invertebrate hemocyte responses to immunological stimuli. The response of hemocytes from the white river crayfish *Procambarus zonangulus* to stimulation by fungal cell walls (zymosan A) were recently measured by FCM (Cardenas *et al.*, 2000). Changes in physical characteristics were assessed using forward- and side-scatter light parameters, and viability was measured by two-color fluorescent staining with calcein-AM and ethidium homodimer 1. The main effects of zymosan A on crayfish hemocytes were reduction in cell size and viability. Adding trypsin inhibitor in reaction mixtures further delayed the reduction in hemocyte size and cell death, thereby indicating that a proteolytic cascade played a key role in generating signal molecules which mediate these cellular responses.

Also LPS from Gram-negative bacteria strongly stimulate hemocytes from *P. zonangulus in vitro* (Cardenas *et al.*, 2004). FCM revealed that treating hemocytes with LPS caused a conspicuous and reproducible decrease in cell size as compared to control hemocytes. This physical modification was accompanied by a reduction in hemocyte viability, that was assessed as described above. The onset of cell size reduction was gradual and occurred prior to cell death. Interestingly, hemocytes treated with LPS from *Salmonella minnesota* without the Lipid A moiety (detoxified LPS) decreased in size without a reduction of viability, while the addition of trypsin inhibitor to the LPS treatments caused noticeable delays in cell size and viability changes. It was concluded that crayfish hemocytes react differently to the polysaccharide and lipid A moieties of LPS, where lipid A is cytotoxic and the polysaccharide portion is stimulatory. These effects concur with the general pattern of mammalian cell activation by LPS, thereby indicating common innate immune recognition mechanisms to bacterial antigens between cells from mammals and invertebrates.

### FCM reveals cellular responses to stress such as environmental pollution

The use of invertebrates as bioindicators in environmental monitoring is of relevant interest for its practical implications. Tunicates are filter feeding marine invertebrates that are susceptible to

environmental contamination by toxic metals and polyaromatic hydrocarbons (Radford *et al.*, 2000). Their immune reactions are profoundly affected by exposure to tributyltin (TBT) and copper, both of which are components of marine antifouling paints. Immunofluorescence labeling with an anti-hemocyte monoclonal antibody whose binding to plasma membrane was revealed by FCM demonstrated that the antigenic structure of the circulating hemocyte population was substantially affected by TBT and copper. Antigen-positive hemocytes were also found to accumulate in the pharyngeal papillae of TBT-exposed tunicates, confirming that exogenous metals can have profound effects on tunicate hemocytes.

The effects of mercuric chloride and methylmercury on the phagocytic capacities of *M. arenaria* hemocytes were evaluated (Fournier *et al.*, 2001). Clams were exposed to single metal in water for up to 28 days at different concentrations ( $10^{-9}$  to  $10^{-5}$  M), and phagocytic activity of hemocytes was determined by uptake of fluorescent microspheres and FCM. The phagocytic capacity is clearly proportional to ingestion of these particles, and thus to the total fluorescence that can be detected from each cell. Clams exposed to high concentrations died by day 7 of exposure, while the viability of hemocytes was decreased only in clams exposed for 28 days to a 10 times lower concentration. A significant decrease in phagocytic activity of hemocytes was also present, and a clear correlation was established between body burden of mercury and effects on phagocytic activity of hemocytes.

Recently, the presence of immunoreactive inducible nitric oxide synthase molecules (ir-iNOS) has been demonstrated in the *L. dispar* IPLB-LdFB cell line. In these cells, ir-iNOS were induced after 18 hour-incubation with sodium nitroprusside (SNP). The increase in NO provoked by SNP in turn induced apoptosis, that was further increased by N-acetyl-L-cysteine. Apoptosis was quantified by FCM through the identification of nuclei showing the typical hypodiploid peak present in cells with less DNA content, after staining with the fluorescent probe propidium iodide (Ottaviani *et al.*, 2001). The analysis of apoptosis by FCM is a very simple assay, that can be performed in almost all cells from all species, since the basic principle is that of staining DNA with a stoichiometric fluorescent molecule. Changes in fluorescence, along with modifications of the physical parameters of the cell, typically occur in those that lose DNA, as during apoptosis.

### Conclusions and perspectives on analysis of invertebrate cells by FCM

A variety of FCM techniques currently exist that allow analysis of almost all cellular functions and parameters, with all of the possible advantages of this approach. From this point of view, until now the world of invertebrates has received a poor attention, and is almost completely virgin territory for more research. Since invertebrate cells are obviously eukaryotic, researchers have indeed the possibility to use all of the methodologies that have been developed for studies on mammalian and human cells. Similarly, almost all reagents and fluorescent dyes that have

been developed to investigate different cellular activities can be successfully used in invertebrates. Even if a variety of functional parameters have still to be investigated, the few data available already provide relevant information not only on the phenotype or the physical characteristics of a given invertebrate cell, but also on its capacity to perform a given function or to respond to a given stress. Considering the relevant importance that comparative studies are assuming, either for the importance of these biological models and their low cost, and the relevant decrease in the cost of flow cytometers (that fortunately are becoming always more affordable), it is easy to predict that in the next few years we will assist in an unprecedented development of cellular studies in the field invertebrate biology and immunology.

### References

- Amen RI, Aten JA, Baggen JM, Meuleman EA, de Lange-de Klerk ES, Sminia T. Trichobilharzia ocellata in *Lymnaea stagnalis*: a flow cytometric approach to study its effects on hemocytes. *J. Invertebr. Pathol.* 59: 95-98, 1992.
- Barcia R, Cao A, Arbeteta J, Ramos-Martinez JI. The IL-2 receptor in hemocytes of the sea mussel *Mytilus galloprovincialis* Lmk. *IUBMB Life.* 48: 419-423, 1999.
- Bihari N, Hamer B, Jaksic Z, Fafandel M, Micic M, Batel R. Application of alkaline elution, Fast Micromethod and flow cytometry in detection of marine contamination. *Cell Mol. Biol. (Noisy-le-grand).* 48: 373-377, 2002.
- Bihari N, Micic M, Batel R, Zahn RK. Flow cytometric detection of DNA cell cycle alterations in hemocytes of mussels (*Mytilus galloprovincialis*) off the Adriatic coast, Croatia. *Aquat. Toxicol.* 64: 121-129, 2003.
- Cao A, Ramos-Martinez JI, Barcia R. In vitro effects of LPS, IL-2, PDGF and CRF on haemocytes of *Mytilus galloprovincialis* Lmk. *Fish Shellfish Immunol.* 16: 215-225, 2004.
- Cardenas W, Dankert JR, Jenkins JA. Flow cytometric analysis of crayfish haemocytes activated by lipopolysaccharides. *Fish Shellfish Immunol.* 17: 223-233, 2004.
- Cardenas W, Jenkins JA, Dankert JR. A flow cytometric approach to the study of crustacean cellular immunity. *J. Invertebr. Pathol.* 76: 112-119, 2000.
- Caselgrandi E, Kletsas D, Ottaviani E. Neutral endopeptidase-24.11 (NEP) deactivates PDGF- and TGF-beta-induced cell shape changes in invertebrate immunocytes. *Cell Biol. Int.* 24: 85-90, 2000.
- Cooper EL, Cossarizza A, Suzuki MM, Salvioli S, Capri M, Quaglino D, Franceschi C. Autogeneic but not allogeneic earthworm effector coelomocytes kill the mammalian tumor cell target K562. *Cell Immunol.* 166: 113-122, 1995.
- Cooper EL, Kauschke E, Cossarizza A. Digging for innate immunity since Darwin and Metchnikoff. *Bioessays.* 24: 319-333, 2002.
- Cossarizza A, Cooper EL, Quaglino D, Salvioli S, Kalachnikova G, Franceschi C. Mitochondrial mass and membrane potential in coelomocytes from the earthworm *Eisenia foetida*: studies with fluorescent probes in single intact cells. *Biochem. Res. Commun.* 214: 503-510, 1995.
- Cossarizza A, Cooper EL, Suzuki MM, Salvioli S, Capri M, Gri G, Quaglino D, Franceschi C. *Earthworm leukocytes* that are not phagocytic and cross-react with several human epitopes can kill human tumor cell lines. *Exp. Cell Res.* 224: 174-182, 1996.
- Dolbear F, Gratzner H, Pallavicini MG, Gray JW. Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. *Proc. Natl. Acad. Sci. USA* 80: 5573-5577, 1983.
- Fomelli F, Minervini F, Mule G. Cytotoxicity induced by nivalenol, deoxynivalenol, and fumonisin B1 in the SF-9

- insect cell line. *In Vitro Cell Dev. Biol. Anim.* 40: 166-171, 2004.
- Fournier M, Pellerin J, Clermont Y, Morin Y, Brousseau P. Effects of in vivo exposure of *Mya arenaria* to organic and inorganic mercury on phagocytic activity of hemocytes. *Toxicology* 161: 201-211, 2001.
- Franceschi C, Cossarizza A, Monti D, Ottaviani E. Cytotoxicity and immunocyte markers in cells from the freshwater snail *Planorbarius corneus* (L.) (Gastropoda pulmonata): implications for the evolution of natural killer cells. *Eur. J. Immunol.* 21: 489-493, 1991.
- Harper DM, Flessas DA, Reinisch CL. Specific reactivity of leukemia cells to polyclonal anti-PCB antibodies. *J. Invertebr. Pathol.* 64: 234-237, 1994.
- Komiyama K, Kauschke E, Cooper EL. Evidence for a perforin-like cell mediated cytotoxicity by earthworm coelomocytes. In: EL Cooper, A Beschin and M Bilej (ed), IOS Press. Amsterdam, The Netherlands, 73-81, 2002.
- Komiyama K, Okawa M, Miki Y, Ohkubo M, Moro I, Cooper EL. Non-specific cellular function of *Eisenia fetida* regulated by polycyclic aromatic hydrocarbons. *Pedobiologia.* 47: 717-723, 2004.
- Micic M, Bihari N, Labura Z, Muller WE, Batel R. Induction of apoptosis in the blue mussel *Mytilus galloprovincialis* by tri-n-butyltin chloride. *Aquat. Toxicol.* 55: 61-73, 2001.
- Mottier V, Siaussat D, Bozzolan F, Auzoux-Bordenave S, Porcheron P, Debernard S. The 20-hydroxyecdysone-induced cellular arrest in G2 phase is preceded by an inhibition of cyclin expression. *Insect Biochem. Mol. Biol.* 34: 51-60, 2004.
- Ottaviani E. Immunorecognition in the gastropod molluscs with particular reference to the freshwater snail *Planorbarius corneus* (L.) (Gastropoda, Pulmonata). *Boll. Zool.* 59: 129-139, 1991.
- Ottaviani E, Barbieri D, Malagoli D, Franchini A. Nitric oxide induces apoptosis in the fat body cell line IPLB-LdFB from the insect *Lymantria dispar*. *Comp. Biochem. Physiol.* 128B: 247-254, 2001.
- Ottaviani E, Cossarizza A. Immunocytochemical evidence of vertebrate bioactive peptide-like molecules in the immuno cell types of the freshwater snail *Planorbarius corneus* (L.) (Gastropoda, Pulmonata). *FEBS Lett.* 267: 250-252, 1990.
- Ottaviani E, Cossarizza A, Ortolani C, Monti D, Franceschi C. ACTH-like molecules in gastropod molluscs: a possible role in ancestral immune response and stress. *Proc. R. Soc. Lond. B Biol. Sci.* 245: 215-218, 1991.
- Ottaviani E, Franchini A, Barbieri D, Klefsas D. Comparative and morphofunctional studies on *Mytilus galloprovincialis* hemocytes: presence of two aging-related hemocytes stages. *It. J. Zool.* 65: 349-354, 1998.
- Ottaviani E, Franchini A, Caselgrandi E, Cossarizza A, Franceschi C. Relationship between corticotropin-releasing factor and interleukin-2: evolutionary evidence. *FEBS Lett.* 351: 19-21, 1994.
- Ottaviani E, Franchini A, Cossarizza A, Franceschi C. ACTH-like molecules in lymphocytes. A study in different vertebrate classes. *Neuropeptides* 23: 215-219, 1992.
- Ottaviani E, Nappi AJ, Vass E. Resistance of the insect cell line IPLB-LdFB to salsolinol-induced apoptosis. *Arch. Insect Biochem. Physiol.* 49: 1-9, 2002.
- Ottaviani E, Petraglia F, Montagnani G, Cossarizza A, Monti D, Franceschi C. Presence of ACTH and beta-endorphin immunoreactive molecules in the freshwater snail *Planorbarius corneus* (L.) (Gastropoda, Pulmonata) and their possible role in phagocytosis. *Regul. Pept.* 27: 1-9, 1990.
- Quaglino D, Cooper EL, Salvioli S, Capri M, Suzuki MM, Ronchetti IP, Franceschi C, Cossarizza A. Earthworm coelomocytes in vitro: cellular features and "granuloma" formation during cytotoxic activity against the mammalian tumor cell target K562. *Eur. J. Cell. Biol.* 70: 278-278, 1996.
- Radford JL, Hutchinson AE, Burandt M, Raftos DA. Effects of metal-based environmental pollutants on tunicate hemocytes. *J. Invertebr. Pathol.* 76: 242-248, 2000.
- Salehzadeh A, Akhka A, Cushley W, Adams RL, Kusel JR, Strang RH. The antimetabolic effect of the neem terpenoid azadirachtin on cultured insect cells. *Insect Biochem. Mol. Biol.* 33: 681-689, 2003.
- Ulrich W. Aneuploid and polyploid cellular DNA heterogeneity in insect cell material of diptera species analyzed by flow cytometry. *Z. Naturforsch.* 45: 1027-1030, 1990.
- White MK, Miosky D, Flessas DA, Reinisch CL. The expression of an adhesion-related protein by clam hemocytes. *J. Invertebr. Pathol.* 61: 253-259, 1993.