

Flow cytometry based techniques to study testicular acidophilic granulocytes from the protandrous fish gilthead seabream (*Sparus aurata* L.)

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Abbreviations: BW, body weight; BSA, bovine serum albumin; SR–2, steroid–free synthetic serum; PBS, phosphate–buffered saline; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FSC, forward scatter; SSC, side scatter; TSB, trypticase soy broth; FC, flow cytometry; ANOVA, one–way analysis of variance; mAb, monoclonal antibody; IL–1 β , interleukin–1 β .

ABSTRACT

The gilthead seabream is a protandrous seasonal breeding teleost that is an excellent model for studying the testicular regression process which occurs in both seasonal testicular involution and sex reversion. Little is known about the cell types and the molecular mechanisms involved in such processes, mainly because of the lack of appropriate methods for testis dissociation, and testicular cell isolation, culture and functional characterization. We have previously reported that gilthead seabream acidophilic granulocytes infiltrate the testis at post-spawning stage, settle close to the spermatogonia and accumulate intracellular interleukin-1 β . In this paper, we report several flow cytometry based assays which allow to establish the role played by gilthead seabream testicular acidophilic granulocytes and permits their quantification.

INTRODUCTION

Spermatogenesis is a complex process in which primary spermatogonia divide and differentiate into spermatozoa. In fish, spermatogenesis appears to proceed in a similar fashion to that observed in mammalian vertebrates. However, the seminiferous

tubules of mammalian vertebrates contain several successive generations of germ cells, all of which depend on the same Sertoli cell (1), while the fish testes are characterized by a cystic structure in which all the germ cells belonging to the same cyst are at the same spermatogenetic stage and progress synchronously surrounded by a cohort of Sertoli cells (2). This structure means that the fish testis is an excellent system for studying the

regulation of spermatogenesis. Recently, it has been suggested that testicular macrophages play an important role in the mammalian testicular function (3). However, in fish, the cells present in the testicular connective tissue are not well characterized, and neither is their role in the regulation of spermatogenesis or in the morphological changes that the fish testis undergoes throughout the reproductive cycle. The gilthead seabream (*Sparus aurata* L.) is a protandrous seasonal breeding teleost that permits the seasonal regression of the testis and sex reversion to be studied. To date, very few cell dissociation, culture and functional assays have been successfully established to study spermatogenesis in fish (4, 5). In this study, we have developed several techniques that, when used together with a specific monoclonal antibody (mAb), allow us to characterise and quantify the functional activities of gilthead seabream testicular acidophilic granulocytes throughout the reproductive cycle.

MATERIALS AND METHODS

Fish

Healthy specimens of mature male gilthead seabream, with body weight (BW) of 100 g were obtained in November, 2002, from Predomar, S.L. (Carboneras, Spain). The fish were kept at the Spanish Oceanographic Institute (Mazarrón, Murcia), in 14 m³ running-seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquaria volume/hour) at natural temperature and photoperiod, and fed twice with a commercial pellet diet (Trouvit, Burgos, Spain). The gonads were removed from the specimens and processed as described below. The animal treatment procedure applied was under considerations of the Bioethical Committee of the University of Murcia.

Testicular cell suspension

The testes were dissociated using a modified protocol from Loir and Sourdain (6). The organ was removed and placed on sbt-L15 medium [L-15 medium (Gibco) adjusted to gilthead seabream serum osmolarity (353.33 mosmol) with 0.35% NaCl (Sigma), supplemented with 20 mM HEPES (Gibco), 3 mM NaHCO₃ (Sigma), 112 mg/l L(+)-lactic acid (Sigma), 1 mg/l glutathione (Sigma), 0.5% gelatin (Sigma), 2 nM CuCl₂ (Sigma), 6 mM Na₂SeO₃ (Sigma), 1 nM MnSO₄ (Sigma), 100 units/ml penicillin and 100 mg/ml streptomycin (P/S, Biochrom)]. The testes were cut and incubated for 5 hours in dissociation medium [Hank's medium (Hank's balanced salt solution without calcium supplemented with 20 mM HEPES, 5 mM NaHCO₃, 5 mg/l glutathione, 2 nM CuCl₂, 6 mM Na₂SeO₃, 1 nM MnSO₄, 5.5 mg/l α -tocopherol acetate (vitamin E, Sigma), 112 mg/l L(+)-lactic acid, 110 mg/l pyruvate (Gibco), essential amino acids (Gibco), non essential amino acids (Gibco), vitamin mixture (Gibco), 0.35% NaCl (Sigma), 30 nM L-ascorbic acid (vitamin C, Sigma), 2 mM glutamine (Gibco), 2% steroid-free synthetic serum (SR-2) (Serum Replacement 2, Sigma) with 1.1 g/l collagenase (Sigma)]. One hour after the start of incubation 20 μ g/ml of DNase I (Sigma) was added.

After dissociation, the testis pieces were incubated overnight in sbt-L15 medium supplemented with 1% bovine serum albumin

(BSA, Sigma), 3 mg/l all trans-retinol acetate (vitamin A, Sigma), 1 mg/l vitamin E, 2 mM glutamine and 2% SR-2. Afterwards, the testis pieces were forced through a 100 μ m nylon mesh and the cell suspension obtained was filtered through a 65 μ m nylon mesh, washed, counted using a Newbauer chamber and adjusted to 5x10⁵ cells/ml in sbt-L15 medium.

Immunofluorescence

Aliquots of 5x10⁵ testicular cells were washed in flow cytometry (FC) buffer [phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS) and 0.05% sodium azide] and incubated for 30 minutes on ice with 100 μ l of a mAb specific to gilthead seabream acidophilic granulocytes (G7) (7), at the optimal dilution of 1:100 in FC buffer. After being washed, cell suspensions were incubated for 30 minutes on ice with 50 μ l of fluorescein isothiocyanate (FITC)-labelled anti-mouse F(ab')₂ fragments of goat antibody (Sigma) at the optimal dilution of 1:100 in FC buffer. Cells were then washed twice and the findings were depicted in the form of two-parameter forward scatter (FSC) and side scatter (SSC) dot plots and green fluorescence (FL1) histograms by using a fluorescence-activated cell sorter (Becton Dickinson). The specificity of staining was checked by using a mAb isotype standard. Each G7 staining was carried out in duplicate.

Labelling of *Vibrio anguillarum* with FITC

The fish pathogenic bacterium *Vibrio anguillarum* (strain R-82, serotype 01) was grown overnight in a trypticase soy broth (TSB) with 1% NaCl and 50 μ g/ml FITC (Sigma) at 25°C in a light-protected environment. After labelling, free FITC was removed by washing 3 times in PBS and the bacteria were inactivated by heating for 20 minutes at 60°C, adjusted to 10⁹ bacteria/ml and then stored at -80°C.

Phagocytosis assay

The phagocytosis of *V. anguillarum* by gilthead seabream testicular cells was studied by FC using a modified protocol from Esteban *et al.* (8) and by electron microscopy. Briefly, aliquots of 5x10⁵ testicular cells in sbt-L15 medium were left untreated or treated with 500 μ g/ml of colchicine (Sigma) for 2 hours at 25°C. Afterwards, testicular cells were mixed with 10⁷ FITC-labelled bacteria, centrifuged at 300g for 5 minutes, resuspended and incubated for 60 minutes at 25°C in a light-protected environment. After the incubation period, the phagocytosis was stopped with ice-cold PBS and the fluorescence of non-ingested bacteria was quenched with a solution of 0.4% trypan blue in PBS. The number of green fluorescent cells (phagocytic cells) was analysed by FC, as described above. Aliquots of cells challenged with *V. anguillarum* for periods ranging from 30 to 90 minutes were also processed for electron microscopy (see below) using 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) as fixative. Each phagocytosis assay was carried out in duplicate.

Viability assay

Aliquots of testicular cell suspensions were diluted in 200 μ l PBS containing 40 μ g/ml propidium iodide. The number of red fluorescent cells (dead cells) from triplicate samples was analysed by FC.

Light and electron microscopy

Small pieces of testis were fixed in Bouin solution, embedded in Paraplast Plus (Sherwood Medical) and sectioned at 5 μ m. After dewaxing and rehydration, the sections were stained with haematoxylin-eosin and examined with a Axiolab (Zeiss) light microscope.

For electron microscopy, samples were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 hours at 4°C, then postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 1 hour at 4°C and embedded in Epon. Ultrathin sections were obtained with a Reichert-Jung ultramicrotome, contrasted with uranyl acetate and lead citrate and examined with a Zeiss EM 10C electron microscope.

Statistical analysis

All the analysis were made using cells from at least four different fish. Data were analysed by one-way analysis of variance (ANOVA) and unpaired Student's t-test to determine difference between groups. A quantitative study of the FC results was made by using the statistical option of the Lysis Software Package (Becton Dickinson).

RESULTS AND DISCUSSION

In this study, conventional FC based techniques were used to clarify the cell population involved in testis regression and germ cell removal. In the protandrous fish gilthead seabream, the tight and highly specialised intercellular unions established in the testis may complicate testis dissociation, for which reason enzymatic and mechanical dissociation methods were used to obtain a testicular cell suspension. After the enzymatic treatment, the cells must be allowed to recover overnight in a highly enriched medium. Medium osmolarity was adjusted to that of gilthead seabream serum by adding 0.35% NaCl. Using this technique, cell viability was around 80%, as assayed by propidium iodide staining.

We analysed the testes of gilthead seabream during the resting stage, a reproductive stage in which spermatogonia is the main cell type in the germinal compartment of the testis (Fig. 1). At this stage, spermatogenetic activity is limited to spermatogonia recrudescence after testicular involution and the resorption of the

remaining germ cells during the post-spawning stage. The testicular cells are heterogeneous in size and complexity, and it is difficult to distinguish populations from FSC and SSC parameters. Moreover, the testis is formed of changeable germ cell populations, which increase in number and decrease in size as the spermatogenesis proceeds. Therefore, we only divided the testicular cell suspension into two regions (R1 and R2) according to cell granularity (SSC) (Fig. 2A).

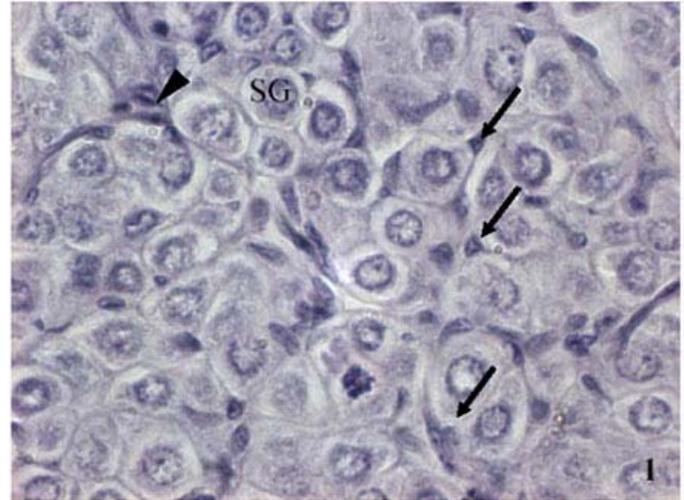


Fig. 1: General view of gilthead seabream testis at the resting stage stained with haematoxylin-eosin. Note that the main cell type is the spermatogonia (SG) and that spermatogonia are surrounded by Sertoli cells (arrows). Arrow head, interstitial tissue. x100.

In contrast to germinal compartment cells, the testicular connective cells have not been well characterised in fish. More specifically, the relative amount of the different leucocyte types and their function in the fish testis are unknown. However some microscopical observations have pointed to the appearance of macrophages, acidophilic granulocytes and lymphocytes in the testicular connective tissue of some teleosts in different stages of the reproductive cycle, mainly during post-spawning (9-14). We have previously reported that gilthead seabream acidophilic granulocytes infiltrate the testis at post-spawning, settle close to the spermatogonia and accumulate intracellular interleukin-1 β (IL-1 β) (15). However, the abundance and the precise role of this cell type is unknown. Therefore, we applied several FC based techniques to characterise and quantify testicular acidophilic granulocytes, using a specific mAb (G7) previously produced in our lab (7). Taking into account that the G7 antibody recognises an acidophilic granulocyte-specific membrane bound protein, ice-cold incubation and inclusion of sodium azide are extremely important in order to avoid antigen uptake after antibody recognition. The G7 antibody only recognised $1 \pm 0.5\%$ of the cells in the testicular suspension of gilthead seabream at the studied stage, but interestingly, the acidophilic granulocytes (G7⁺ cells) belong exclusively to the R2 region where they represented around 30% of the cells (Figs. 2B and C).

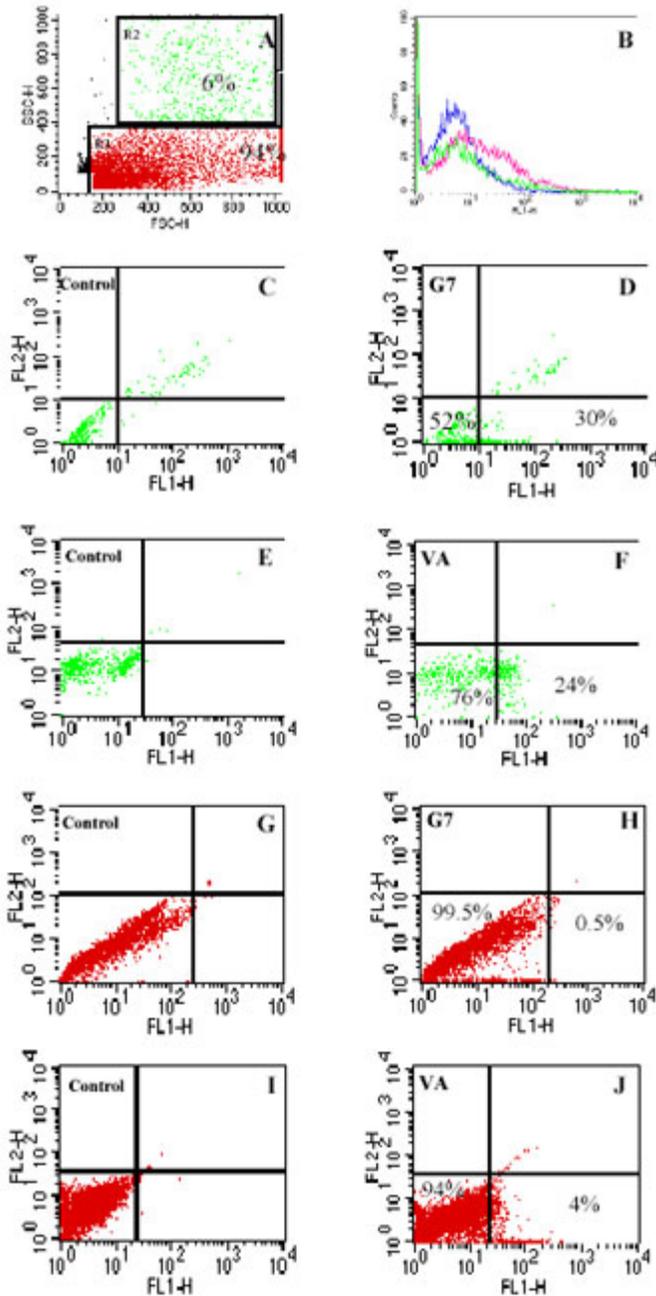


Fig. 2: Flow cytometry analysis of gilthead seabream testicular cell suspension. (A) Representative dot blot of FSC (size) *vs* SSC (granularity). (B) Representative FL1 (green fluorescence) histograms of testicular cells incubated in the absence (pink histogram) or presence (blue histogram) of 500 $\mu\text{g}/\text{ml}$ colchicine for 2 hours and then challenged with FITC-labelled *V. anguillarum* for 1 hour. Non-challenged cells (green histogram) were also shown for comparison. (C-J) Representative dot blots of FL1 (green fluorescence) *vs* FL2 (red fluorescence) of cells present in region R1 (G-J) and region R2 (C-F) stained with G7 (D, H), challenged with FITC-labelled bacteria (VA) (F, J) or negative controls (C, E, G, I). Note that both R1 and R2 regions had a phagocytic cell population (F, J) and that only cells belonging to the R2 region were stained with G7 (D).

We were able to quantify the percentage of phagocytic cells present in the testicular cell suspensions by FC. For the data to be reliable, the assay has to be conducted on ice after the

incubation time to avoid bacterium digestion and fluorescence quenching by phagosomal acidification. Cells treated with colchicine, a drug that blocks the polymerisation of microtubules, was used as appropriate negative control. Cells pretreated with colchicine and then challenged with FITC-labelled bacteria showed a similar fluorescence to non-challenged cells, indicating that trypan blue successfully quenched the fluorescence of non-ingested bacteria (Fig. 2D). Applying this technique, $10 \pm 3\%$ of testicular cells showed phagocytic activity against *V. anguillarum*. Notably, phagocytic cells are present in both R1 and R2 regions, where they represent around 4% and 24%, respectively, in each region (Figs. 2E and F). These data suggest the existence of two cell populations (non granulated and granulated) with phagocytic activity in the testis of the gilthead seabream. Sertoli cells are considered to be the main cell population with phagocytic capacity involved in the elimination of germ cells in the testis (13, 16-18). Our data suggest that, if it is the case, not all Sertoli cells show phagocytic activity against *V. anguillarum* during the resting stage of the reproductive cycle, since the percentage of phagocytic cells obtained was very low compared with the estimated number of Sertoli cells in the gilthead seabream testis during the resting stage (Fig. 1). The similar percentage of both acidophilic granulocyte and granulated phagocytic cells present in testicular cell suspension, together with the fact that gilthead seabream head kidney acidophilic granulocytes show high phagocytic activity against *V. anguillarum* (7), prompted us to use electron microscopy to identify the cell types showing phagocytic activity against this bacterium in the gilthead seabream testis. Strikingly, cells with ultrastructural features of gilthead seabream acidophilic granulocytes (7, 19, 20) were never observed to contain ingested bacteria (Fig. 3A), suggesting that the cells of R2 region with phagocytic activity were not acidophilic granulocytes. However, there is a testicular cell population that was able to ingest the bacterium (Fig. 3B), indicating that the testis is not depleted in defense against bacterial infection. Unfortunately, the lack of specific markers against gilthead seabream macrophages and testicular somatic cells means that it was, up to day, impossible to determine the nature of the cell type showing phagocytic activity against *V. anguillarum* in the testis during the resting stage. Our data do not exclude, however, a phagocytic role for testicular acidophilic granulocytes during other reproductive stages since the fish testis is a changeable organ with tightly regulated cell activity.

To conclude, our data support that fish testicular cells may be cultured and analysed by FC (21), although several modifications of the currently available methods are needed, depending on the fish salinity adaptation and the complexity of the testis of each species studied. To our knowledge, this is the first time that testicular acidophilic granulocytes have been characterised and quantified in a testicular cell suspension, by using FC based techniques. Moreover, we show that it is possible to use FC to study testicular cell phagocytic activity, a function that may be involved in the abrupt morphological changes that occur in this organ during a very tight timing schedule in the reproductive cycle.

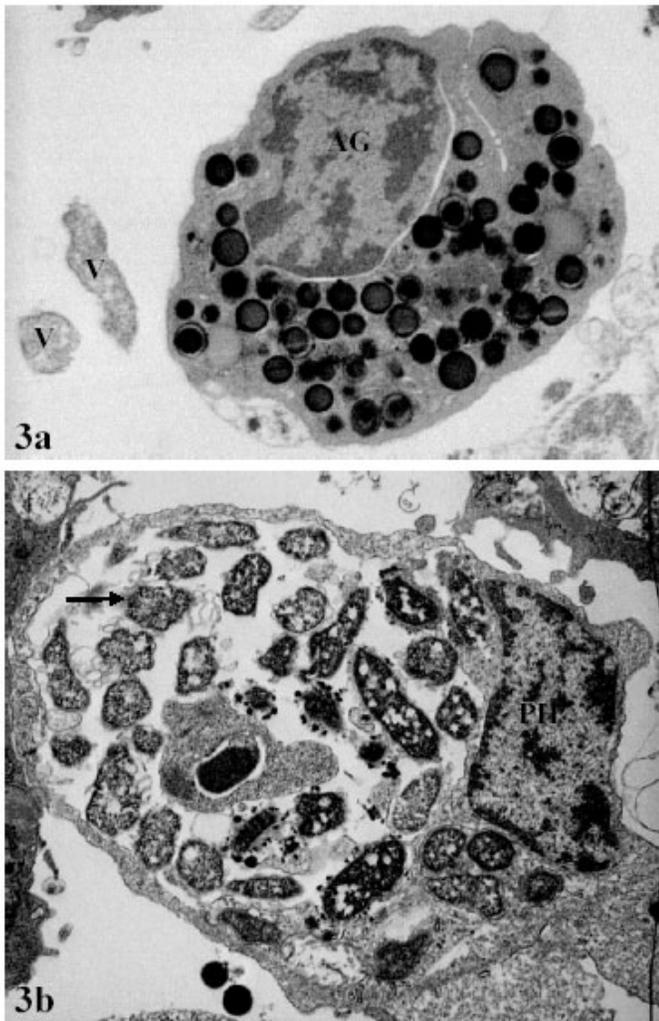


Fig. 3: Transmission electron microscopic pictures of an acidophilic granulocyte (AG) with numerous cytoplasmic granules which does not phagocytose the bacterium (V) (a) and a phagocytic cell (PH) with a large phagosome containing numerous bacteria (arrow) (b) from a testicular cell suspension incubated with *Vibrio anguillarum* for 90 minutes. (a) x4000, (b) x5000.

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PROTOCOLS

Isolation of testicular cells

1. Remove the testis and place in sbt-L15 medium.
2. Cut the testis into approximately 1 mm² pieces and place 1 gr testis in 6 ml dissociation medium [Hank's medium containing 1.1 g/l collagenase (Sigma) previously sterilized by u.v. irradiation]. Incubate the testicular pieces for 1 hour at 25°C with slow shaking.
3. Add 20 µg/ml DNase I (Sigma) previously sterilized by u.v. irradiation and dissolved in a small volume of Hank's medium. Incubate for 4 hours at 25°C with slow shaking.
4. After incubation, remove the dissociation medium, and incubate the testis pieces with sbt-L15 medium supplemented with 1% bovine serum albumin (BSA, Sigma), 3 mg/l all trans-retinol acetate (vitamin A, Sigma), 1 mg/l vitamin E, 2 mM glutamine and 2% SR-2 overnight at 25°C with slow shaking.
5. Force the testis pieces through a nylon mesh of 100 µm size using sbt-L15 medium, rinse cell suspension by centrifuging for 5 minutes at 300 g and filter through a nylon mesh of 65 µm size. Wash the cell suspension twice with sbt-L15 medium by centrifuging for 5 minutes at 300 g, count using a Newbauer chamber and adjust to 5x10⁶ cells/ml in sbt-L15 medium.

Immunofluorescence

1. Wash aliquots of 5x10⁵ testicular cells in ice-cold FC buffer by centrifuging for 5 minutes at 300 g.
2. Incubate the cells with 100 µl of G7 mAb diluted 1:100 in FC buffer for 30 minutes on ice.
3. Wash the cells twice in ice-cold FC buffer by centrifuging for 5 minutes at 4°C and 300 g.
4. Add 50 µl with a fluorescein isothiocyanate (FITC)-labelled anti-mouse F(ab')₂ fragments of goat antibody (Sigma) diluted 1:100 on ice-cold FC buffer and incubate for 30 minutes on ice in a light-protected environment.
5. Wash the cells twice in ice-cold FC buffer by centrifuging for 5 minutes at 4°C and 300 g in a light-protected environment.
6. Resuspend the cells in 200 µl ice-cold FC buffer and analyse by FC.

Phagocytosis assay

1. Labelling of *Vibrio anguillarum* (strain R-82, serotype 01)

- a. Grow the bacteria in 20 ml trypticase soy broth (TSB, Pronadisa) with 1% NaCl and 50 µg/ml FITC (Sigma) overnight at 25°C in a light-protected environment.
- b. Wash three times in phosphate buffer saline (PBS) by centrifuging for 10 minutes at 1000 g.
- c. Inactivate the bacteria heating at 60°C for 20 minutes.
- d. Wash once in PBS by centrifuging for 10 minutes at 1000 g.
- e. Measure OD at 600 nm and adjust to 10⁹ cfu/ml (0.5 OD units - 10⁸ cfu/ml).
- f. Aliquot and store at -80°C.

2. Flow cytometry (FC) analysis

- a. Incubate aliquots of 5x10⁵ testicular cells with or without 500 µg/ml colchicine (Sigma) during 2 hours at 25°C.
- b. Mix the aliquots with 10⁷ FITC-labelled bacteria and centrifuge 5 minutes at room temperature and 300 g.
- c. Resuspend by pipetting and incubate 60 minutes at 25°C in a light-protected environment.
- d. Add 500 µl of ice-cold PBS and place the samples on ice in order to stop the phagocytosis.
- e. Add 50 µl of ice-cold trypan blue (0.4% in PBS).
- f. Mix gently, acquire and analyse by FC.

3. Electron microscopy analysis

- a. Challenge 10⁷ testicular cells with 2x10⁸ heat-inactivated bacteria and centrifuge 5 minutes at room temperature and 300 g.
- b. Resuspend by pipetting and incubate from 30 to 90 minutes at 25°C.
- c. Add 500 µl of ice-cold PBS and place the samples on ice in order to stop the phagocytosis.
- d. Centrifuge 5 minutes at 300 g.
- e. Resuspend in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) during 1 hour at 4°C.
- f. Process for conventional electron microscopy.

Viability assay

1. Dilute aliquots of 5×10^5 testicular cells in 200 μ l PBS containing 40 μ g/ml propidium iodide.
2. Analyse by FC.

Buffers and media

1. *sbt-L15 medium*

Reagent	Final concentration
L-15 powder (Gibco)	12.18 g/l
NaCl (Sigma)	35 g/l
HEPES (Gibco)	4.76 g/l
NaHCO ₃ (Sigma)	248 mg/l
L(+)-lactic acid (Sigma)	112 mg/l
Glutathione (Sigma)	1 mg/l
Gelatine (Sigma)	0.5 g/ml
CuCl ₂ (Sigma)	0.25 μ g/l
Na ₂ SeO ₃ (Sigma)	5 μ g/l
MnSO ₄ (Sigma)	0.15 μ g/l
Penicillin/Streptomycin (P/S, Biochrom)	100 units/ml/100 mg/ml

Measure and adjust the pH to 7.4.

2. *Hank's medium*

Reagent	Final concentration
Hank's powder (Sigma)	12.18 g/l
HEPES	4.76 g/l
NaHCO ₃	413 mg/l
Glutathione	5 mg/l
CuCl ₂	0.25 μ g/l
Na ₂ SeO ₃	5 μ g/l
MnSO ₄	0.15 μ g/l
α -tocopherol acetate (Sigma)	5.5 mg/l
L(+)-lactic acid	112 mg/l
Pyruvate (Gibco)	110 mg/l
Essential aminoacids 50x (Gibco)	1x
Non-essential aminoacids 100x (Gibco)	1x
Vitamin mixture 100x (Gibco)	1x
NaCl	3.5 g/l
L-ascorbic acid (Sigma)	30 nM
Glutamine (Gibco)	2 mM
Serum Replacement 2 (Sigma)	20 ml/l

Measure and adjust the pH to 7.4.

3. *Phosphate buffer saline (PBS)*

- Mix 2.67 g Na₂HPO₄, 0.828 g NaH₂PO₄ and 17.55 g NaCl in 2 l distilled water and adjust the pH to 7.4.

4. *FC buffer*

- PBS containing 2% fetal calf serum (FCS) and 0.05% sodium azide.

5. *Cacodylate buffer 0.1 M*

- Dilute 21.4 g sodium cacodylate in 1 l of distilled water and adjust the pH to 7.2.

6. *Bouin solution*

- Mix 71 ml picric acid saturated solution, 24 ml 40% formol and 5 ml acetic acid.