Assessment of the Cell Viability of Cultured Perkinsus marinus (Perkinsea), a Parasitic Protozoon of the Eastern Oyster, Crassostrea virginica, Using SYBRgreen–Propidium Iodide Double Staining and Flow Cytometry

PHILIPPE SOUDANT, a, b FU-LIN E. CHU b and ERIC D. LUND b

a LEMAR, UMR 6539, IUEM-UBO, Technopole Brest-Iroise, Place Nicolas Copernic, 29280 Plouzané, France, and 
b Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, Virginia 23062, USA

ABSTRACT. A flow cytometry (FCM) assay using SYBRgreen and propidium iodide double staining was tested to assess viability and morphological parameters of Perkinsus marinus under different cold- and heat-shock treatments and at different growth phases. P. marinus meront cells, cultivated at 28 °C, were incubated in triplicate for 30 min at −80 °C, −20 °C, 5 °C, and 20 °C for cold-shock treatments and at 32 °C, 36 °C, 40 °C, 44 °C, 48 °C, 52 °C, and 60 °C for heat-shock treatments. A slight and significant decrease in percentage of viable cells (PVC), from 93.6% to 92.7%, was observed at −20 °C and the lowest PVC was obtained at −80 °C (54.0%). After 30 min of heat shocks at 40 °C and 44 °C, PVC decreased slightly but significantly compared to cells maintained at 28 °C. When cells were heat shocked at 48 °C, 52 °C, and 60 °C heavy mortality occurred and PVC decreased to 33.8%, 8.0%, and 3.4%, respectively. No change in cell complexity and size was noted until cells were heat shocked at ≥44 °C. High cell mortality was detected at stationary phase of P. marinus cell culture. Cell viability dropped below 40% in 28-day-old cultures and ranged 11–25% in 38 to 47-day-old cultures. Results suggest that FCM could be a useful tool for determining viability of cultured P. marinus cells.

Key Words. Cell growth, cell mortality, culture phases, cytomorphology, temperature tolerance.

Numerous methodologies have been described and employed for assessments of number and viability of in vitro cultured cells and microorganisms. While microscopic methods are simple and accommodating, they are not effective for studies that require numeration and cell viability measurement of a large number of samples. In this context, analysis by flow cytometry (FCM) has advantages over microscopic approaches, since it allows multi-parametric analyses of a single cell and can be performed on a large numbers of cells in a very short time (100–1,000 cells/s) (Barbesti et al. 2000; Dias and Lima 2002; Grégori et al. 2001; Kato and Bowman 2002).

In aquatic ecology, FCM is routinely employed for determination of abundance, viability, and activity of viruses (Marie et al. 1999), bacteria (Bernard et al. 2001; Bunthof et al. 1999; Lebaron, Catala, and Parthuisot 1998; Lebaron et al. 2001; Mortimer, Mason, and Gant 2000; Roth et al. 1997; Williams et al. 1998), phytoplankton (Grégori et al. 2002; Marie et al. 1997; Marie et al. 2000; Olson, Zettler, and Durand 1993), and planktonic protozoans (Lindström, Weiss, and Stadler 2002; Parrow and Burkholder 2002; Wong and Whiteley 1996). Rapid and reliable FCM methods have also been developed to enumerate and to evaluate growth, viability, infectivity, and metabolic activities of gastroenteric protozoan parasites, such as Cryptosporidium spp. and Giardia spp. (Campbell, Robertson, and Smith 1992; Foster et al. 2003; Kato and Bowman 2002; Medema et al. 1998; Schupp and Erlandsen 1987; Vesey et al. 1994; see also Quintero-Betancourt, Peele, and Rose 2002 for review), and of intra-erythrocytic protozoans, such as Plasmodium falciparum (Srivastava, Rottenberg, and Vaidya 1997), Babesia bovis (Wyatt, Goff, and Davis 1991), Leishmania amazonensis (Bertho, Cysne, and Coutinho 1992; Guinet et al. 2000), and Theileria sergenti (Yagi et al. 2000).

Perkinsus marinus is one of the two important parasites of the Eastern oyster, Crassostrea virginica, and presently the disease caused by this parasite is most prevalent among Eastern oyster populations along the east and Gulf coasts of the United States. Temperature and salinity are the two important environmental factors affecting the progression and geographic distribution of P. marinus. Four life-cycle stages—trophozoites (meronts, merozoites), prezoosporangium (hypnozoites), zoosporangium, and biflagellated zoospor—have been identified and described (Perkins 1966, 1988). Trophozoites are considered to be the primary agents for disease transmission (Chu 1996; Perkins 1988). In culture, cell number and morphology of P. marinus vary depending on nutrient composition and culture conditions (see review, Villalba et al. 2004). Different life-cycle stages and different sizes of the same stage (e.g. schizonts or mother cells and different sizes of trophozoites) can be found in the same culture (Sunila, Hamilton, and Dunagan 2001).

Recently, FCM protocols using nucleic acid double staining, SYBRgreen I or II and propidium iodide (PI), have been developed to assess abundance and viability of cultivated bacteria (Barbesti et al. 2000), bacteria in natural environments (Grégori et al. 2001), and oyster hemocytes (Delaporte 2005). SYBR green I or II stain the nucleic acids of both live and dead cells while PI stains only nuclei of cells that have lost their membrane integrity (dead cells and cells with damaged membrane).

The objectives of the present study were to test the feasibility of using SYBRgreen–PI double staining with FCM to measure cell number, viability, size, and complexity of cultured P. marinus meront cells under different temperature-shock treatments and at different growth phases. Additionally, the assay, which uses 3, (4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium and phenazine ethosulfate (MTS/PMS) to assess cell viability and proliferation based on dehydrogenation of oxidizable substrates by dehydrogenase of live cells coupled with the reduction of the tetrazolium dye, was utilized as a comparison for the viability measurement by FCM. Due to the great importance of temperature on disease progression in the field (Burreson and Ragone-Calvo 1996), the effects on P. marinus meronts in vitro exposure to high and low temperature may provide insight into the epidemiology of this disease.

MATERIALS AND METHODS

In vitro cell culture. Meronts of P. marinus were cultivated as previously described (Chu et al. 2002) in the medium defined by La Peyre, Faisal, and Burreson (1993). The medium was prepared with artificial seawater (ASW) and adjusted to an osmolarity of 590 (equivalent to salinity 20 psu, Lund, Chu, and Harvey 2004), then sterilized by 0.2 μm filtration, and stored at 4 °C until use. Meronts were inoculated (1 × 10^6 cells/ml) in
10-ml aliquots of medium in T-10 tissue culture flasks and cultured at 28 °C.

Experiments

Cold and heat shocks. Seven-day-old meront cell cultures grown at 28 °C were sampled and distributed (2 ml) in equivalent numbers (3.1 ± 0.2 × 10⁸ cells/ml for the cold shock and 4.3 ± 0.3 × 10⁸ cells/ml for the heat shock) in polypropylene tubes (capacity = 15.0 ml). Cells were incubated for 30 min in triplicate for cold-shock treatments at −80 °C, −20 °C, 5 °C, and 20 °C and for heat-shock treatments at 32 °C, 36 °C, 40 °C, 44 °C, 48 °C, 52 °C, and 60 °C. The meront cultivation temperature of 28 °C was used as the control (reference) temperature. After incubation (30 min) was terminated and as samples were cooled down or warmed up to room temperature, all cell suspensions were filtered through an 80-µm mesh to eliminate large pieces of debris. Then, 100 µl of the resulting cell suspension from each replicate of each treatment were pipetted into a tube containing 500 µl of 20 psu artificial sea water (ASW), equivalent to the osmolarity of the P. marinus culture medium.

Meront cells at different ages. Meront cell cultures 7, 14, 25, 28, 38, 42, and 47 days old were sampled and processed as aforementioned.

Analysis of viability, size, and complexity of P. marinus meront cells by FCM. Two nuclease fluorescent dyes were used simultaneously for this assay: the permeant SYBRgreen I (ex = 497 nm, em = 520 nm; Molecular Probes, Eugene, OR) and the impermeant propidium iodide (PI, ex = 535 nm, em = 617 nm; Sigma, St. Louis, MO). The permeant SYBRgreen I purchased from Molecular Probes was a 10,000× concentrate stock solution; no information on either mass or molar concentration was provided from the manufacturer. SYBR green stains both live and dead cells and PI stains only cells that have lost membrane integrity. Thus, SYBR fluorescence on the yellow-fluorescence detector (FL1) differentiates live and dead P. marinus cells from other particles (e.g. cell debris) present in the medium and from instrument “noise,” while PI allows the detection of dead cells of P. marinus on the red-fluorescence detector (FL3).

Dead meront cells, prepared by killing the meront cells with absolute ethanol, were used as negative controls for viability measurement. Briefly, 1 ml of cell suspension was transferred slowly to 4 ml of absolute ethanol at −20 °C while vortexing at top speed. Cells were incubated at −20 °C for 15 min, pelleted by centrifugation, and then reuspended in 1 ml of 0.2-µm filtered ASW at room temperature. The cells were allowed to rehydrate for 15 min prior to staining.

Fifteen minutes prior to analysis, 6 µl SYBRgreen I (final concentration: 1/10,000 of the commercial stock solution) and PI (final concentration: 10 µg/ml) were added to the cell suspension. FCM measurements of double-stained meront cells were carried out using a Beckman Coulter® EPICS® Altra™ flow cytometer (Miami, FL) connected to a computer using PC Expo32 as data acquisition software. Optical alignment and stability were monitored daily using 10-µm diam. fluorescent flow-count fluorospheres (Coulter PN6605359). To adjust the FCM settings fixed (dead) and live cell samples stained with SYBRgreen I, PI or SYBRgreen I+PI were assayed. Cells with single stain (SYBRgreen I or PI) allowed adjusting the electronic compensations between detectors (FL1 versus FL3). Non-stained cell samples were also assayed to ensure that cell auto-fluorescence remained below 10 on both detectors.

Meront cell samples from cold- and heat-shock treatments, and at different ages (see above in the “experiments” section) were double stained with SYBRgreen I+PI. Generally, acquisition time of 30 s was found sufficient to obtain between 2,000 and 10,000 analyzed cells. In all the assays, meront cell concentrations between 0.2 and 1.0 × 10⁸ cells/ml were analyzed. Data acquisition was made in logarithmic mode (four decades).

Treatment of the FCM data was performed with the software WinMDI v. 2.8 (Joseph Trotter®). Biparametric representations (density plots) of ethanol-fixed cells (Fig. 1A) and live cells (Fig. 1D) showed that the P. marinus cells are homogeneously distributed in terms of Forward Scatter (FSC) and Side Scatter (SSC) parameters and were easily defined by a circular region. FSC, corresponding to the diffraction light on the small angle (detected in line with the incident light source), is proportional to the size. SSC, corresponding to the diffraction light on the right angle, is proportional to the cell complexity or granular content. Geometric means of these parameters were used to characterize, in a relative manner, cell size and complexity of P. marinus, and expressed as Arbitrary Units (AU). After gating cells on R1 of the SSC-FSC density plot, FL1 fluorescence using a histogram allowed visualization of the SYBRgreen-stained particles, which are either live or dead cells (Fig. 1B, E). After secondary gating of the SYBRgreen-stained cells on R2 of the FL1 histogram, the FL3 fluorescence histogram allowed visualization of the SYBR- and PI-stained cells, which were considered dead (Fig. 1C, F).

The FL1 histogram of R1-gated ethanol-fixed cells showed a large peak of green fluorescence corresponding to DNA containing P. marinus cells (Fig. 1B). These cells accounted for 95.4% of the events presented in R1. After gating on both R1 and R2, the FL3 histogram of the ethanol-fixed cells also showed a large peak corresponding to cells that incorporated PI (Fig. 1C). All cells with a red fluorescence above 30 were considered as dead cells, presumably due to the loss of membrane integrity. The M2 marker showed that 99% of the ethanol-fixed cells were dead. The SYBRgreen peak of fresh (non-fixed) cells prepared from the same culture was similar to that obtained with fixed cells (Fig. 1E). However, the PI peak was smaller than for the fixed cells (cf. Fig. 1C, E), and was used to measure the percentage of dead cells in fresh cell preparations (Fig. 1F). In this sample, cells with a red fluorescence above 30 (AU) accounted for 40.2% of SYBR-stained cells. Therefore, it is estimated that the assayed culture contained 40% dead cells (or 60% of viable cells). To estimate cell concentration of the cell suspension analyzed by FCM, the flow rate of the instrument was measured using a known concentration of fluorospheres (fluorescent flow-count fluorospheres, Coulter PN6605359).

MTS/PMS assay. The effects of temperature shocks on the viability of P. marinus meronts were also assessed using the MTS/PMS assay. The MTS/PMS assay assesses cell viability and proliferation based on dehydrogenation of oxidizable substrates by dehydrogenase of live cells coupled with the reduction of the tetrazolium dye. It indirectly measures the number of viable cells since the amount of mitochondrial dehydrogenase present in the samples is directly proportional to the number of viable cells. This assay has been applied to measure viability and proliferation of other cultured cells, including P. marinus (Dungan and Hamilton 1995).

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyphenyl)-2-(4-sulphonylphenyl)-2H-tetrazolium, inner salt (MTS) was obtained from Promega (CellTiter 96 AQ, Cat.#G11111, Madison, WI) and phenazine methosulfate (PMS) was obtained from Sigma (P9625, St. Louis, MO). MTS and PMS reagents were prepared according to manufacturer’s instructions (Promega, CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Technical Bulletin #169, Promega Corporation, Madison, WI). Briefly, 20 µl of the MTS/PMS working solution were added to 100 µl of cell suspension in a flat-bottomed 96-well microtiter plate. The micro-plate was then incubated for 3 h at 28 °C in the dark. The absorbance (optical density, OD) of formazan, the reduction product of MTS/PMS, was measured at 450 nm using a Tecan GENios
Fig. 1.  Forward Scatter and Side Scatter density plot (A and D), yellow-green fluorescence histogram (B and E), and red fluorescence histogram (C and F) of ethanol-fixed and live meronts of *Perkinsus marinus*, respectively, stained with SYBRgreen+PI.
micro-plate reader (Maennedorf, Switzerland). Instrument control and data acquisition by MS-Excel™ were provided by the computer software XFLUOR4 V 4.50 (Tecan, Maennedorf, Switzerland). Samples used for the MTS/PMS assay were from the same stocks as those measured by FCM and assayed at two dilutions: 1 and 1/5. The OD value of the 1/5 dilution was used when OD values of non-diluted cell suspensions were above 1.0, and corrected accordingly with the dilution factor. As the number of cells used for the assay was identical at all temperatures, the percentage of viable cells at different temperature shocks was expressed as the percentage of the OD measured at the control/reference temperature of 28 °C according to the formula OD at different temperatures/OD at 28 °C × 100%.

Statistical analysis. Data were analyzed statistically using the software STATGRAPHICS v.4.1. One factor analysis of variance (ANOVA) was utilized to compare the differences (1) of percentage of viable cells, (2) of morphological characteristics (size and complexity as measured, respectively, by the FSC and SSC parameters) under different temperature-shock treatments, (3) of the cell concentration, (4) of the percentage of viable cells, and (5) of morphological characteristics (FSC and SSC) of meront cultures at different ages. Differences were considered significant when P-value was < 0.05. When results were significant at the P < 0.05, Fisher’s least significant difference (LSD) procedure (multiple range test) was used to discriminate among the means.

RESULTS

FCM analysis of *P. marinus* cells stained with SYBRgreen or PI and SYBRgreen+PI. When the FL1 fluorescence intensity of cells stained with SYBRgreen I+PI was compared to cells stained with only SYBRgreen I (Fig. 2A), FL1 was reduced by 2.5-fold. In contrast, FL3 fluorescence intensity of cells stained with SYBRgreen I+PI was increased by 2.7-fold when compared with cells stained with only PI (Fig. 2B). The observed phenomena are called “fluorescence resonance energy transfers” or more trivially “quenching.” It occurs between two nucleic acid fluorochromes when they are both closely bound to the nucleic acid.

Cell viability, size, and complexity after cold shocks. Cell viability measured by FCM (Fig. 3A). Meront cell viability was expressed as percent of viable cells (PVC). PVC of 7 day-old meronts (in suspension) incubated at the control/culture temperature of 28 °C for 30 min was 93.7%. Incubation of meronts at 20 °C and 4 °C for 30 min did not change the cell viability compared to the control. But a slight decrease of PVC (92.7%) was observed after 30 min at −20 °C and the lowest PVC was obtained after 30 min at −80 °C (54.0%). Thirty-minute temperature shocks at 20 °C, 5 °C, −20 °C, and −80 °C did not change the mean *P. marinus* cell size and complexity as assessed by the FSC and SSC parameters, respectively. Size ranged from 81.5 to 88.7 AU and complexity ranged from 85.7 to 91.0 AU (Table 1).

Cell viability measured by MTS/PMS assay (Fig. 3B). With the MTS/PMS assay, incubation of meronts at 20 °C and 5 °C for 30 min did not reduce cell viability compared to those incubated at the control temperature, but PVC decreased significantly, almost by half, after a temperature shock at −20 °C for 30 min. This is different from the result obtained using FCM: only a slight decrease of PVC was noted after a temperature shock at −20 °C for 30 min (cf. Fig. 3A, B). With a temperature shock of −80 °C, meront viability was significantly reduced by 5-fold compared to the control reference temperature of 28 °C and by 3-fold compared to the viability at −20 °C. Overall, the percentage of viable cells as measured by the MTS/PMS assay followed the same pattern as the percentage of viable cells (PVC) measured by FCM. These two measurements were significantly (P < 0.001) correlated [R² = 0.964; PVC(MTS/PMS) = 6.91 + 0.956 × PVC(FCM)].

Viability, size, and complexity after heat shocks. Cell viability measured by FCM (Fig. 4A). The PVC was slightly higher than 93% from 28 °C to 36 °C. Slight but significant decreases in PVC occurred at 40 °C and 44 °C: the PVC at 40 °C and 44 °C were 91.5% and 84.4%, respectively. PVC was reduced to 33.8% after heat shock of 48 °C for 30 min. When meronts were heat shocked at 52 °C and 60 °C for 30 min, heavy mortality occurred and PVC decreased to 8.0% and 3.4%, respectively. Cell complexity was relatively stable between 28 °C and 44 °C and ranged from 60.4 to 64.8 AU. Similarly, meront sizes were constant, ranging from 68.7 to 70.9 AU between 28 °C and 40 °C. However, both cell complexity and size changed when meronts were heat shocked at ≥ 44 °C. Cell size decreased, but cell complexity increased significantly with increasing incubation temperatures (Table 2).

Cell viability measured by MTS/PMS assay (Fig. 4B). The percentage of viable cells was ≥ 95% between 32 °C and 40 °C (Fig. 4B), but the PVC decreased significantly when meronts were heat shocked at 44 °C and further decreased to 10.1% at 60 °C. Similarly to the cold-shock treatment, percentages of viable cells measured by the MTS/PMS assay paralleled those

![Image](https://via.placeholder.com/150)
measured by FCM ($R^2 = 93.1; P<0.001; PVC({MTS/PMS}) = 1/((0.0999+0.0009) \times PVC(FCM))$).

**Meront viability and morphology at different growth phases.** Cell concentration (Table 3) increased rapidly between day 7 and day 14 from $2.9 \pm 1.2 \times 10^6$ to $8.1 \pm 0.9 \times 10^6$ cells/ml. There was no significant change in cell concentrations from day 14 to day 47 with the exception of day 25 at which a cell concentration of $5.9 \pm 1.8 \times 10^6$ cells/ml was recorded. The PVC decreased steadily and significantly from 93.6% at 7 d to 79.0% at 25 d (Fig. 5), and then reached a plateau varying at 11.2–24.5%.

Table 1. Cell size and cell complexity of *Perkinsus marinus* meronts at different cold-shock temperatures, as measured, respectively, by Forward Scatter for cell size and Side Scatter parameters for cell complexity (mean, SD, n = 3).

<table>
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<th>Temperature (°C)</th>
<th>Size (AU)</th>
<th>Complexity (AU)</th>
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<td></td>
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<td>SD</td>
</tr>
<tr>
<td>28</td>
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<td>-80</td>
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Different letters denote significantly different at $P<0.05$ among treatments.

Table 2. Cell size and cell complexity of *Perkinsus marinus* meronts at different heat-shock temperatures, as measured, respectively, by Forward Scatter for cell size and Side Scatter parameters for cell complexity (mean, SD, n = 3).

<table>
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<th>Complexity (AU)</th>
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<td>69.3a</td>
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</tr>
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<td>52</td>
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<td>1.6</td>
</tr>
<tr>
<td>60</td>
<td>50.3a</td>
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Different letters denote significantly different at $P<0.05$ among treatments.
between 38 and 47 d. Morphological parameters (Table 3) were also significantly different among culture ages. Cell size and complexity decreased rapidly from 7 to 28 d, and then no significant change occurred afterward.

**DISCUSSION**

Results from the present study indicate that it is feasible to assess cell viability, size, and complexity of meronts of *P. marinus* using FCM with double nucleic acid stains (i.e. SYBRgreen I and propidium iodide, PI). Treating *P. marinus* meronts with 100% ethanol apparently killed almost all the cells. Nearly 100% of the ethanol-treated cells took up the PI base on FCM analysis. When combined with SYBRgreen, the magnification of PI fluorescence (2.7-fold) was due to fluorescence resonance energy transfer (also called quenching) from SYBRgreen I to PI. This phenomenon occurs between two nucleic acid fluorochromes when they are both closely bound to the nucleic acid (Barbesti et al. 2000). However, the quenching from SYBRgreen I to PI obtained with the nus complex bound to the nucleic acid (Barbesti et al. 2000). However, quenching from SYBRgreen I to PI obtained with the *P. marinus* cells was not as high as those observed when applied to bacteria (Barbesti et al. 2000; Grégori et al. 2001). This difference in quenching intensity between bacteria and *P. marinus*, a parasitic protozoan, may reflect the difference in chromosomal structures between prokaryote cells and unicellular eukaryote cells.

Experimental infections revealed that prevalence and intensity of *P. marinus* in oysters is positively correlated with temperature (Chu 1996; Chu et al. 1993; Chu and La Peyre 1993). *P. marinus* proliferates and develops rapidly at high temperatures between 20°C and 30°C (Chu and Greene 1989; Chu, Soudant, and Lund 2003; Dungan and Hamilton 1995; Gauthier and Vasta 1995; Volety and Chu 1997). Dungan and Hamilton (1995) showed that *P. marinus* could proliferate between 10°C and 40°C, while no proliferation occurred at 4°C. On the other hand, Gauthier and Vasta (1995) did not report significant differences in proliferation between 4°C and 15°C. Lower metabolic activity in meronts was also observed at lower temperature. Chu et al. (2003) demonstrated that the uptake and assimilation of exogenous lipids and triacylglycerol lipase activities was drastically reduced in meronts of *P. marinus* grown for 7 d at 10°C as compared to those cultivated at 18°C and 28°C.

*Perkinsus marinus* appeared to be more sensitive to heat shock than to cold shock. Cold shock of meront cells at 5°C did not produce significant cell death compared to their cultivation temperature of 28°C. A cold-shock treatment of meront cells at -20°C for 30 min only induced 1% increase of cell mortality (percentages of viable cells were 92.6% at -20°C versus 93.6% at 28°C). However, a temperature as low as -80°C killed cells rapidly with a mortality more than 50%.

Also, cold temperature shocks did not cause a change in cell size and complexity as compared to those maintained at 28°C. The observed tolerance to cold temperature in meronts of *P. marinus* was not surprising, since *P. marinus* can be easily cryo-preserved (Dungan and Hamilton 1995; Gauthier and Vasta 1995). Chu and Greene (1989) showed that hyponosome of *P. marinus* could tolerate temperatures as low as 4°C for 4–7 d. The tested heat-shock temperatures of 32°C, 36°C, 40°C, 44°C, 48°C, and 52°C are comparable to those encountered by *P. marinus* within its host oyster in the field. Oysters are ectothermic with body temperatures close to the ambient environment. For those living in the intertidal zone, a rapidly fluctuating environment could experience temperatures as high as 55°C during low-tide exposure (Willson and Burnett 2000).

Indeed, temperatures in the intertidal may exceed the thermal tolerances of *P. marinus* (Bushek et al. 2002). Results of our study are in agreement with the findings of previous studies that temperatures higher than 35°C inhibited the proliferation and induced mortality of *P. marinus* (Dungan and Hamilton 1995; La Peyre and Faisal 1996). As heat-shock temperature rose to 36–40°C, mortality began and increased dramatically from 40°C to 44°C. All these results suggest that *P. marinus* is less thermal tolerant than its host, the Eastern oyster. Galtsoff (1964) reported survival of emersed intertidal oysters at 46–49°C for up to 2–3 h. Ingle et al. (1971) also reported survival of intertidal Gulf oysters at 49.5°C. In a recent study, we noted that no mortality occurred in two different geographic oyster stocks after heat shocks at 41°C and 42°C for 1 h (Encomio and Chu 2003; Encomio 2004).

The significant increase of cell complexity in *P. marinus* after heat shock at temperatures of 48°C and above may be due to protein denaturation as protein denaturation is known to increase cell complexity and light scattering in mammalian models (Le-Pock 2005). Moreover, Tirard et al. (1995) showed that induction of heat shock proteins (Hsp), which are produced to limit protein denaturation, occurred above 46°C in *P. marinus*. These authors also observed that Hsp was produced at a higher temperature of 46°C in *P. marinus* than in *C. virginica*, which was induced from 39°C to 41°C. Based on these observations, they concluded that *P. marinus* was more thermo-tolerant than the oyster. However, one could also argue that an inability to mount a rapid Hsp

**Table 3.** Cell size and cell complexity of *Perkinsus marinus* meronts at different growth phases, as measured, respectively, by Forward Scatter for cell size and Side scatter parameters for cell complexity (mean, SD, n = 2–4).

<table>
<thead>
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<th>Days of culture</th>
<th>Size (AU)</th>
<th>Complexity (AU)</th>
<th>Cell concentration (cell/ml)</th>
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<td>Mean</td>
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<td>Mean</td>
</tr>
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<td>47</td>
<td>46.4c</td>
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</table>

Different letters indicate the significant differences between culture phases.
production in response to temperature shock could result in decreased survival at high temperatures. Additionally, Tirard et al. (1995) did not monitor the viability of the cells on which they measured their Hsp production.

The cell cultures of _P. marinus_ at stationary phase exhibited a surprisingly high cell mortality. Thus, the mortality occurring in aging cell cultures may result in bias in studies aiming to examine cellular and metabolic activities of different phases of the growth cycle of _P. marinus_ cultures. We also observed that the cell mortality at stationary phase varied among batches of cell culture (unpubl. data). Thus, cell viability measurement is recommended for any future cellular and metabolic study.

In addition to directly counting cells employing FCM and microscopy, several bioassays have been described for assessment of cell viability and/or proliferation in in vitro culture. These assays include measurements using neutral red uptake, reduction of a tetrazolium dye, and uptake of radioactively labeled thymidine (³H-thymidine) (e.g. Borenfreund and Puerner 1985; Buttte, McCubrey, and Owen 1993; Dungan and Hamilton 1995; Gillis et al. 1978; Green, Reade, and Ware 1984; Volety and Chu 1997). These assays provide quantitative measurements of live cells based on their biological functions: uptake of neutral red via pinocytosis (neutral red assay); dehydrogenation of oxidizable substrates by dehydrogenase coupled with the reduction of the tetrazolium dye (MTS/PMS assay); and uptake of ³H-thymidine into cellular DNA. The results of cell viability measurement employing FCM are in good agreement with those obtained from the MTS/PMS assay, based on their highly significant correlation. Nevertheless, these two assays assess different biological functions related to cell viability: mitochondrial dehydrogenase for the MTS/PMS assay and loss of membrane integrity for the FCM assay. The MTS/PMS assay does not directly measure cell viability, but provides an estimation of cell viability via the measurement of mitochondrial NADH- and NADPH-dependent dehydrogenase activities in living cells. It can be noted that the MTS/PMS assay detected a greater effect on parasite viability compared with the FCM assay for cold shocks at −20 °C to −80 °C and for heat shocks at 44–48 °C. So, it is likely that metabolic activities such as mitochondrial dehydrogenase are firstly affected by thermal stress and that loss of membrane integrity occurs with the increased intensity of the stress. Employing the SYBrGreen–PI double staining facilitates the direct measurements of living and dead cells, and cells with damaged membranes, with additional information concerning cell size and complexity changes. The time-consuming microscopic trypan blue exclusion assay also directly gives measurement of cell viability. However, in addition to the potential problem of inefficient uptake of the dye by the nucleus of dead meronts, considering the morphology (e.g. eccentric vacuole, numerous lipid droplets, and small, slightly convex centrifugally located nucleus) and size (approximately 2–5 μm) of meronts (Perkins 1996), it may be difficult to obtain unbiased/correct counting between live and dead cells.

In summary, the present study demonstrated that it is feasible to employ FCM to assess cell viability, cell size, and cell complexity of meronts of _P. marinus_ exposed to different temperature shock treatments and at different growth phases. Thus, FCM assay could be used routinely to monitor cell viability and morphological changes in _P. marinus_ cultures under different culture conditions.

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