

## Rapid Assessment of Genotoxicity by Flow Cytometric Detection of Cell Cycle Alterations

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### Abstract

Flow cytometry is a convenient method for the determination of genotoxic effects of environmental pollution and can reveal genotoxic compounds in unknown environmental mixtures. It is especially suitable for the analyses of large numbers of samples during monitoring programs. The speed of detection is one of the advantages of this technique which permits the acquisition of  $10^4$ – $10^5$  cells per sample in 5 min. This method can rapidly detect cell cycle alterations resulting from DNA damage. The outcome of such an analysis is a diagram of DNA content across the cell cycle which indicates cell proliferation,  $G_2$  arrests,  $G_1$  delays, apoptosis, and ploidy.

Here, we present the flow cytometric procedure for rapid assessment of genotoxicity via detection of cell cycle alterations. The described protocol simplifies the analysis of genotoxic effects in marine environments and is suitable for monitoring purposes. It uses marine mussel cells in the analysis and can be adapted to investigations on a broad range of marine invertebrates.

**Key words** Genotoxicity assessment, Cell cycle alterations, DNA content, Flow cytometry, Marine mussel, *Mytilus galloprovincialis*

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### 1 Introduction

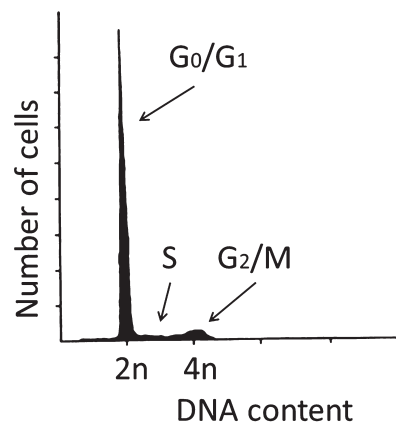
Analysis of DNA content and cell cycle alterations by flow cytometry is widely used in detection of genotoxic and/or xenobiotic effects in different cell types from vertebrates and invertebrates. The DNA content of cells is assessed with the help of the stoichiometrically binding DNA dyes, so that the emitted fluorescence is proportional to the DNA amount. The most widely used dye is 4',6-diamidino-2-phenylindole (DAPI). DAPI has a strong A-T base preference and yields DNA histograms with coefficients of variation (CVs) lower than that obtained using other dyes. Additionally, staining with DAPI is less affected by the state of chromatin condensation compared to other DNA dyes.

Flow cytometric DNA analysis is performed to determine the percentage of cells in each phase of the cell cycle and to evaluate

cell cycle alterations in cellular populations. As a rule, all normal diploid cells (non-replicating  $G_0$  cells and cells in  $G_1$  phase of cell cycle) in the same eukaryotic organism should have the same DNA content. This quantity is usually expressed as  $2n$ . DNA synthesis during the S phase of the cell cycle results in an increase in cellular DNA content, which reaches  $4n$  at the end of S phase and remains at this value during the  $G_2$  phase and during mitosis (M phase). After the completion of mitosis the original cell is replaced by two daughter cells, each with DNA content of  $2n$ . A typical DNA content distribution across a cell cycle obtained by flow cytometry is shown in Fig. 1. The analyzed cells fall into three categories: (1) Cells in  $G_0$  or  $G_1$  phase, i.e., with an unreplicated complement of DNA; (2) Cells in  $G_2$  or M phase with a fully replicated complement of DNA (twice the  $G_1$  DNA content); (3) Cells in S phase, with an intermediate amount of DNA.

The distribution of cells in Fig. 1 indicates that there are more cells in the  $G_0/G_1$  phase than in the  $G_2/M$  phase showing that  $G_0/G_1$  is longer than  $G_2/M$  in this population. The DNA content distribution always exhibits some variance in the  $G_0/G_1$  peak, which may be due to staining procedures, to instrumental errors, and/or to cell-to-cell differences in DNA content.

The obtained DNA content distribution is analyzed automatically by the software package supplied with the flow cytometer. The analysis includes the calculation of CV, i.e., the standard deviation of the distribution for the diploid peak divided by the peak mean, and the percentage of cells in  $G_0/G_1$ , S, and  $G_2/M$  phases during the cell cycle. Higher CVs can often result from chromosomal aberrations caused by clastogenic agents—mutagens inducing disruption or breakages of chromosomes [1]. A larger CV may also be due to the partial inclusion of an aneuploidy peak in the



**Fig. 1** Typical DNA content histogram obtained with flow cytometry

diploid peak [2]. There is an established link between the increase in CV and both chemical [3] and radiation exposure [4]. Increases in CVs have been reported for erythrocytes of turtles exposed to low-level radiations in effluent ponds of nuclear power plants [4], for blood samples of green frogs exposed to pesticides [5], for vertebrates exposed to radionuclides and other mutagenic chemicals [4, 6], for blood in fish from Chernobyl-contaminated ponds [7], and, more recently, for clams collected at polluted sites of Sagueny Fjord, Canada [8].

Flow cytometry is useful in determining the pollutant-induced genetic damage in marine invertebrate populations. Flow cytometric analysis was successfully used in studies of DNA alterations in different organs of the marine mussel *Mytilus galloprovincialis* [9]. Mussels of the genus *Mytilus* are among the most common of the marine molluscs, constituting an important element of both the ecology of coastal waters and the economy as food and fouling organisms. They accumulate various contaminants from the surrounding water and serve as sensitive bioindicators of coastal water quality. Because hemolymph and gills of marine mussels respond first to genotoxic agents, they are used for the monitoring of environmental conditions. Cell cycle alterations in the hemolymph DNA were reported for mussels collected at sampling sites that were under the influence of anthropogenic loads [10] and in mussels treated in vivo with the herbicide 2,4-dichlorophenoxy acetic acid [11]. Induction of internucleosomal DNA fragmentation, i.e., apoptosis, was detected in gill tissue from mussels treated with tri-*n*-butyltin chloride [12]. Thus, the flow cytometric analysis of DNA content in marine mussels can be used as a pollution test in the ecosystem survival studies of polluted areas.

This chapter describes the flow cytometric procedure for the rapid assessment of genotoxicity via detection of DNA content and cell cycle alterations. The sample preparation is shortened and does not require the hemocyte isolation step. The described protocol simplifies the analysis of genotoxic effects in marine environments and is suitable for monitoring studies. It uses mussel *Mytilus galloprovincialis* hemolymph and gills cells in the analysis and can be adapted to investigations on a broad range of marine invertebrates.

The step-by-step description includes the instrument setup and standardization, sample preparation, DNA content measurement, data analysis, and reporting. We also provide several examples of altered DNA content histograms obtained by flow cytometric analysis. The described protocol requires up to 5 min for the acquisition of  $10^4$ – $10^5$  cells (one sample) and allows analysis of more than 50 samples per day. The procedure gives information about several cell cycle alterations in the analyzed samples.

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## 2 Materials

1. Standards: trout and chicken erythrocyte nuclei as indicators of DNA ploidy (commercially available).
2. 4' 6-diamidino-2-phenylindole dihydrochloride (DAPI) staining solution for nonfixed cells (Partec GmbH, Germany).
3. Flow Cytometer (Partec PAS II or Partec PAS III that we used, or other available models).
4. Hemolymph probe (*see Note 1*): add 100  $\mu\text{L}$  of hemolymph ( $1\text{--}2 \times 10^6$  hemocytes per mL of hemolymph) to the 1 mL DAPI staining solution containing 10% of dimethyl sulfoxide (DMSO) and analyze immediately.
5. Gills probe: 1 mg of gills (about  $1 \times 10^6$  cells) gently resuspend in DAPI staining solution; filter through 30  $\mu\text{m}$  filter and analyze immediately.

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## 3 Method

### 3.1 Instrument Setup

Instrument setup varies with manufacturer. However, there are some general principles to observe.

1. Select the LIN channel that is most appropriate for the DNA probe.
2. Set the trigger on the channel detecting the DNA probe.
3. Select parameters to enable doublet discrimination (*see Note 2*).
4. Set a gate to exclude doublets and apply it to the histogram that will display the DNA profile.
5. Make sure the sheath tank is full, as it may help with stability.
6. Make sure the cytometer is clean. Stream disruption will increase the CV (*see Note 3*).
7. Set a low flow rate and dilute cells to a concentration that is appropriate for the DNA probe solution.
8. Make sure the instrument has been optimized by running routine calibration particles.

### 3.2 Standardization

1. Check the performance of the instrument on a daily basis using commercially available fluorescent beads of known CV or DNA standards (*see Note 4*). Any perturbation of the sample stream in the cytometer will increase the CV and for this reason the concentration of cells or nuclei should be kept high (between  $5 \times 10^5$  and  $2 \times 10^6/\text{mL}$ ) and the flow rate low.
2. Check that there is not a partial blockage of the flow cell. Report the CV of the main  $G_0G_1$  peak. Generally, less than 3 is good; greater than 8 is poor.

### 3.3 Sample Preparation

1. Take the hemolymph from the adductor muscle of the mussel (*see Note 5*) and prepare the hemolymph probe (*see Note 6*).
2. Dissect the gills from the mussel and prepare the gills probe.

### 3.4 DNA Content Measurement

1. Analyze the hemolymph or gill probe with the flow cytometer: flow rate 200–400 cells/s, excitation of 100 W mercury lamp, emission 455 nm for the DAPI signal or excitation with UV laser at 359 nm, and emission at 461 nm. Analyze  $2 \times 10^4$  hemocytes or gill cells from each sample. First, make the acquisition of the control sample (stained hemolymph or gills of untreated healthy specimen) and then of the investigated sample.
2. Repeat measurements in triplicates and rerun after adding 10  $\mu$ L of mussel sperm internal standard (*see Note 7*) in order to calculate the DNA index (DI). DI is generally defined as the ratio between the DNA content of a tumor cell and that of a normal diploid cell. In the mussel protocol the DI value is calculated as the ratio between the position of the diploid peak of the investigated cells and that of the mussel sperm DNA (internal standard), as discussed in Subheading 3.5. Data analysis and reporting.
3. Finally, rerun control samples after each individual series (*see Note 8*). Variability due to differences in sample preparations, staining procedures, condition of the mercury lamp, and adjustments of the flow cytometer optical system should be fairly constant (small samples CV standard deviation) (*see Note 9*).

### 3.5 Data Analysis and Reporting

1. Calculate the DI value as the ratio between the position of the  $G_0/G_1$  peak of the affected cell line or tissue and that of normal diploid cells (control), in which case it should be  $1 \pm 0.05$  [2].

The flow cytometer provides a histogram of the DNA content distribution across a cell cycle. Altered DNA content distribution is immediately observable from the histogram. Furthermore, CVs as well as the percentage of cells in each phase of the cell cycle are already provided by the instrument's software package, while the DI value should be calculated. To analyze if the CV reflects the affected cells compare only CVs of normal and symmetrical DNA content histograms (Table 1).

2. In the case of mussels calculate the DI value as the ratio between the position of the diploid peak of the investigated cells and that of the mussel sperm DNA (internal standard) (*see Notes 10 and 11*).

**Table 1**  
**Coefficient of variations for  $G_0/G_1$  peak of mussel hemolymph**

| Mussels  | N  | CV   | Confidence interval |
|--|----|------|---------------------|
| Maricultured   | 30 | 4.59 | 4.22–4.77           |
| Injected with DMSO as a vehicle for different contaminants | 15 | 4.85 | 4.63–5.07           |
| Collected along Adriatic coast                             | 20 | 5.11 | 4.95–5.28           |
| Collected at site under the anthropogenic load             | 10 | 6.87 | 6.04–7.42           |

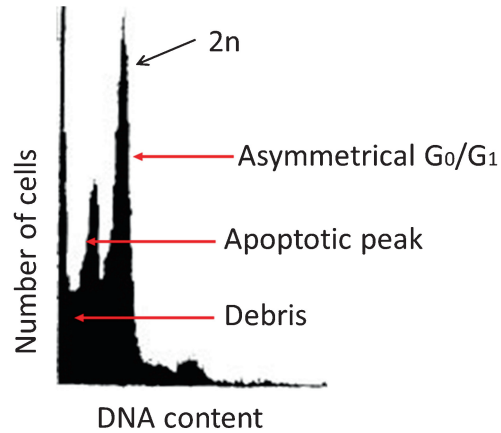
## 4 Notes

1. It is not necessary to isolate and use a particular hemocyte type from mussel hemolymph since hemolymph cell kinetics is uniform although it contains different hemocytes. The reason is low DNA content variation in different hemocyte types, not detectable by flow cytometry.
2. Two nuclei or cells in  $G_1$  of the cell cycle stuck together will have the same DNA content as a single cell in  $G_2$  and the two should be distinguished if the DNA histogram is to reflect accurately the state of the cell cycle. Doublets will give a wider signal than single cells while cells in  $G_2/M$  will give a higher peak signal compared to two clumped cells in  $G_1$  (doublet) but a narrower width. Some instruments are designed to display width against area; other models display peak height against area.
3. The CV as a measure of instrument precision and the peak channel number for a standard set of conditions (laser power, photomultiplier voltage, and gain) should be recorded. Predetermined limit for CV is 2% for calibration particles or stained nuclei since DNA content is so precisely regulated so as to vary by less than 2% from cell to cell in homogeneous, non-dividing populations. Restore the instrument's performance if these fall outside the predetermined limit.
4. Perform the standardization of the instrument and the acquisition of a control sample to distinguish between effects of the instrument setup and effects of genotoxins in the examined cells. During the standardization the following criteria for mussel tissues must be achieved:
  - Acquisition of at least 20,000 nuclei.
  - Low flow rate (100–200 cells/s) for narrow CV.

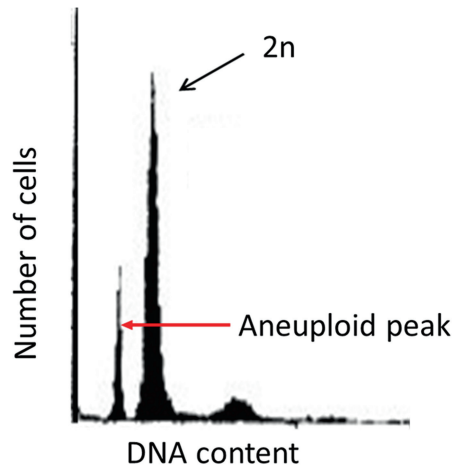
- $G_0/G_1$  peak on a suitable scale in a known channel.
- CV of the  $G_0/G_1$  peak  $5 \pm 0.5\%$ .
- S phase containing at least 200 cells.
- Data containing less than 3% debris.
- $G_0/G_1$  to G2 M ratio between 1.95 and 2.05.

If the above is achieved for control mussel tissues any change in the DNA content histogram, increase in CV, or change of DI value of the analyzed sample could be attributed to the alteration of the cycle caused by genotoxins.

5. Open the mussel valves carefully and just enough to insert the needle of a syringe without damaging the adductor muscle. Slightly insert the needle into the adductor muscle and withdraw 100  $\mu\text{L}$  of hemolymph. It is possible to withdraw up to 600  $\mu\text{L}$  of hemolymph from a mussel 4–5 cm in length. Be careful not to tear the adductor during the process. If necessary, check the hemolymph content for the presence of hemocytes using a microscope.
6. Prepare the hemolymph probe immediately at the site of mussel collection and freeze it in liquid nitrogen for transportation to the laboratory. Store at  $-80^\circ\text{C}$  up to 1 year.
7. As instrument calibration standard use commercially available trout erythrocytes. As an internal fluorescence standard use freshly isolated and fixed mussel sperm, obtained as described in [13]. Briefly, the mussel sperm is resuspended in a buffer-fluorochrome solution. The latter is prepared by adding 10% DMSO to combined solutions A (4 parts) and B (1 part), just prior to use. Solution A: 0.85% w/v NaCl, 0.1 M Tris, 1 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{MgCl}_2$ , 0.2% w/v bovine serum albumin, adjusted to pH 8.0; Solution B: 106 mM  $\text{MgCl}_2$ , 0.6% w/v Triton X-100, 50  $\mu\text{g}/\text{mL}$  DAPI. Samples should be kept frozen at  $-70^\circ\text{C}$  until use.
8. Rerunning the control sample is a necessary step in order to rinse and check the performance of the instrument between sequentially analyzed samples.
9. CVs strongly depend on the type of flow cytometer used for measurements, e.g., for healthy mussel hemolymph the CV of the diploid peak calculated by the software package has been  $3.5 \pm 0.5$  ( $n = 60$ ) and  $4.5 \pm 0.5$  ( $n = 57$ ) for two instrument types we tested (Partec PAS II and Partec PAS III), respectively.
10. For normal, healthy mussel specimens, DI calculated relative to mussel sperm is 1.8–2.0.
11. Examples of altered DNA content histograms obtained by flow cytometric analysis are shown in Figs. 2 and 3. These demon-



**Fig. 2** DNA content histogram of apoptotic mussel hemolymph cells



**Fig. 3** DNA content histogram of aneuploid mussel hemolymph cells

strate two examples of altered DNA profiles obtained for hemolymph of several mussels collected along the Adriatic coast: apoptotic DNA content histogram (Fig. 2) and DNA content histogram that reveal aneuploidy (Fig. 3). Apoptosis is characterized by (1) An asymmetrical  $G_0/G_1$  peak, (2) A decrease of the  $G_2/M$  peak concomitant with the appearance of cells characterized by low DNA content, i.e., below that of the  $G_0/G_1$  peak (so-called apoptotic peak) and (3) Increase in the number of dead cells appearing as debris at the far left side of the DNA content distribution. DNA content distribution describing aneuploidy shows an additional peak on the left side of the diploid peak arising from chromosome loss [14]. Aneuploidy is also specified by the decrease in DI values [15].



For mussel hemolymph, aneuploidy with only hypodiploid DNA content, DI lower than 0.9 was detected in 30.8% of the 146 investigated mussels collected along the Adriatic coast [9].

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