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Quantitative analysis of turbellarian cell suspensions by fluorescent staining with acridine orange, and video microscopy

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ABSTRACT. A combination of methods has been developed for analyzing cell suspensions from turbellarians with respect to cytochemical and morphometric parameters and with special emphasis on the characterization of neoblasts. Tissues were disintegrated by mechanical and enzymatic means. Neoblasts were separated and / or fractionated by different centrifugation protocols in Percoll density-gradients. Staining with acridine orange under conditions denaturing RNA but leaving intact double stranded DNA yielded green fluorescence of DNA and red phosphorescence of RNA. Both light emissions were documented and quantitative image analyses were performed. By computing the integral intensities of green and red light, quantitative measures were gained for DNA and RNA contents. By correlating these light emissions to each other or to cell size, histograms characteristic for given neoblast pools were obtained. The protocol described should facilitate monitoring the heterogeneity of the neoblast compartment as well as studying cellular dynamics during growth, regeneration and other physiological processes.

KEY WORDS: acridine orange, cell suspensions, disintegration of tissues, DNA, *Dugesia tahitiensis*, fluorescence microscopy, image analysis, *Macrostomum*, neoblast fractions, planarians, RNA, video microscopy, Platyhelminthes.

INTRODUCTION

Turbellarians are generally assumed to consist of two compartments from a cell biological point of view: a functional compartment made up of differentiated cells incapable of dividing and a proliferative compartment (BAGUÑÀ, 1998; BAGUÑÀ et al., 1994). In triclad planarians, the latter undergoes considerable variation depending on size, nutritional state and need for regeneration (BAGUÑÀ & ROMERO, 1981). It is built up by a relatively uniform cell type, mostly termed neoblast (BAGUÑÀ, 1981). A high nucleocytoplasmic ratio that may be estimated with phase contrast optics and a strongly basophilic cytoplasm with mitochondria, densely packed ribosomes but no further organelles are the main characteristics of the neoblast. There are various indications for the heterogeneity of this cell population that comprises true stem cells, progenitor cells and early stages of differentiating cells (for a review, see BAGUÑÀ et al., 1994). The number of methods available for analyzing this heterogeneity is rather limited. Their uniform appearance is an obstacle to distinguishing subtypes and finding the true stem cells among neoblasts. Among other criteria, cell kinetic data (BAGUÑÀ et al., 1989), behaviour in density-gradient centrifugation combined with morphological characters (SCHÜRMANN et al., 1998) and ultrastructural traits (HORI, 1992, 1997; RIEGER et al., 1999) have been used for substantiating this heterogeneity. Recent developments include, in addition, the application of bromodeoxyuridine (BrdU) labeling (LADURNER et al., 2000; NEWMARK & SÁNCHEZ ALVARADO, 2000) to follow cell proliferation and monitoring of *vasa* (*vas*)-related transcripts for distinguishing somatic stem cells from germ cells and differentiated somatic cells (SHIBATA et al., 1999). Whereas BrdU should turn out as a label universally applicable, the *vasa*-related genes refer to chromatoid bodies. These are documented well for triclads, but are absent, for example, in the Macrostomidae (RIEGER et al., 1999). It is still an open question if the corresponding gene is expressed in this case.

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A quantitative and relatively rapid cytochemical approach should greatly assist in subtyping neoblasts and monitoring variations within the neoblast pool. In a planarian, DNA amounts surpass the contents equivalent to the G1 phase only in these cells, and their ribosomal RNA forms a prominent fraction. Therefore, correlating DNA and RNA contents with each other and with cell size (volume) and other morphometric parameters such as circularity, elongation factor and circumference could form a basis for characterizing and monitoring the neoblast compartment. For this purpose, fluorescent staining of cell suspensions from *Dugesia tahitiensis* Gourbault 1977 has been combined with quantitative image analysis. Neoblasts from *Macrostomum* n. sp. were analyzed by the same methods.

MATERIAL AND METHODS

Animals

Specimens of *Dugesia tahitiensis* Gourbault 1977 (Turbellaria: Tricladida Paludicola) were taken from a laboratory stock derived from the original collection made for the description of this endemic and purely asexual species (GOURBAULT, 1977). *Macrostomum* n. sp. (Turbellaria: Macrostomorpha), belonging to the *M. tuba* group, was supplied by Prof. Dr. Reinhard Rieger, Innsbruck, who reared a laboratory stock from animals collected in the Adriatic sea near Lignano, Italy (RIEGER et al., 1999). Reagents were obtained from Sigma with a few exceptions cited in the following text. Whenever available, tissue culture grade reagents were applied.

Disintegration (maceration) of tissues

For a high yield of cell suspensions with a low percentage of aggregates (these impede monitoring individual cells in image analysis), former techniques applying a combined mechanical and enzymatic tissue disintegration (SCHÜRMANN et al., 1998) were modified with respect to the enzyme mix. Whole animals starved for one week and kept in 0.02% neomycin sulfate in culture water for at least 24 h were disintegrated in Dounce homogenizers with loosely fitting pestles. Tolerances of 50-70 µm (25-35 µm on each side) were found suitable for *D. tahitiensis* and *Macrostomum* n. sp. Disintegration was performed with ten strokes of the pestle in an ice bath. Isotonic media were applied for both species (see SCHÜRMANN & PETER, 2001, for *Dugesia*). In any case, glucose (10 mM) was added to support the survival of the cells. For *Dugesia*, a first incubation with DNAse (460 U/ml; Sigma DN-25) and hyaluronidase (2100 U/ml; Sigma type V, H-6254) lasted 10 min at 18 ºC. Then, collagenase (600 U/ml, Sigma type IV, C-5138) and its essential cofactor calcium chloride (0.6 mM in the assay) were added and the tissue was resuspended in the homogenizer using the pestle as described above. A further incubation (30 min, 18 ºC) was followed by the addition of EDTA (disodium salt, concentration of 2 mM in the assay), resuspension of cells in the homogenizer as above and an incubation period of 10 min at 18 ºC. DNAse hydrolyzed DNA from damaged cells that would otherwise clump the cells together. By the combined treatment with hyaluronidase and collagenase, the extracellular matrix was degraded sufficiently to avoid the formation of aggregates. For *Macrostomum* n. sp., a medium approximately isotonic to sea-water proved best. It consisted of 250 mM KCl, 250 mM NaCl, 10 mM HEPES buffer (pH 7.4), 2 mM EDTA (disodium salt), 10 mM glucose and 460 U/ml DNAse. Finally, the cell suspension was either washed (see below) and stained for fluorescence microscopy or filtered through a series of meshes with 40, 30, 20 and 15 µm mesh size for separating smaller cells, in particular neoblasts. For cells from *Macrostomum* n. sp., an 8µm net was added. In any case, debris was eliminated by one or several washing steps (centrifuge for 10 min at 500xg and 4 ºC and resuspend) before staining. All media for centrifugation contained 2 mM EDTA and 10 mM glucose. A change of medium was achieved by analogous centrifugation steps. Finally, the cell suspensions were diluted with the last washing medium to yield the desired cell density. By this method, cells with a high viability were obtained. Roughly 80% of the cells filtered through the last net were scored as live (green versus red fluorescence for dead cells) in the LIVE/DEAD Kit L-7013 of Molecular Probes (BELETSKY & UMANSKY, 1990; POOT, 1997). In a pilot experiment, maceration as described by BAGUÑÀ & ROMERO (1981) was combined with fluorescent staining. As cell membranes were permeabilized by this procedure, gradient centrifugation was not applicable.

Gradient centrifugation

Either total cell suspensions or neoblast sub-fractions were used for further analysis. To separate and fractionate neoblasts, one of the protocols described for centrifugation in Percoll gradients (SCHÜRMANN et al., 1998) or a newly developed *in situ* gradient may be used. To run this gradient, 2 ml of the final cell suspension ($2x10^6$ cells per ml or less) were mixed with 8 ml of Percoll solution (Pharmacia) to yield a final density of 1.08. Centrifugation (15-30 min, 32,000xg, 4 ºC) using an angle rotor (Beckman JA-21, 40º) resulted in the formation of a continuous gradient (1.02-1.10) and the simultaneous isopycnic separation of cells according to their buoyant densities. Fractions were collected after perforating the centrifuge tubes with a hypodermic needle, and the Percoll was removed by one to three washing steps (centrifugation at 500xg for 10 min at 4 ºC) with fivefold dilution with medium in each step.

Acridine orange staining

For fluorescent staining, acridine orange (Molecular Probes, high purity grade A-1301) was chosen as a dye intercalating into dsDNA, thereby showing enhanced

green fluorescence (λ_{max}=522 nm) upon excitation at a λ_{max}=502 nm and forming aggregates with polyanions such as ssRNA that emit red light (λ_{max}=638 nm) by phosphorescence. Under proper conditions, DNA is kept in the double stranded form, whereas double stranded RNA regions are denatured to single strands (DARZYNKIEWICZ & KAPUSCINSKI, 1990; WATSON, 1991). Several staining protocols, including such with previous fixation of the cells, were compared with each other (ROMEIS, 1989; TRAGANOS et al., 1977). A two-step equilibrium method with acridine orange present during observation and measurement proved superior, as photobleaching was avoided and the bright fluorescence persisted for at least 12 h when the slides were stored at 4 °C in a moist chamber. The treatment consisted in a detergent pretreatment (0.07% Triton- X100 in the assay at pH 3.0) followed by staining at pH 3.8 with a final dye concentration of approximately 40 µM, in the presence of EDTA and NaCl to selectively denature RNA (TRAGANOS et al., 1977, p. 47). Staining was performed in suspension. Thereafter, slides were prepared with the aid of a cytocentrifuge (Cytorotor in a Heraeus Megafuge 1.0) and viewed with a Leitz Aristoplan microscope equipped with epifluorescence, using the dual band filter XF 53 (Omega Opticals), with bandpasses from about 540-560 nm and 620-640 nm.

Microscopy and image analyses

For documentation and quantitative analysis, the images captured from the microscope by a 3CCD video camera (Hamamatsu C5810) were stored in a PC connected online to the camera. The variable integration time of the camera was set to yield maximal red and green intensities well below the maximum of 255 and thus lying within the linear range of the grey scale. With the image analysis software LUCIA 3.52a (Laboratory Imaging, Prague), red and green light intensities were integrated on a pixel basis and related to cell and nuclear sectional areas. These areas are projections of the largest extension of a cell and comprise all fluorescent (phosphorescent) light emitted from the whole cell or nucleus, respectively. They represent, therefore, the cell volume. The intensities of the red and green light emitted are proportional to the amount of nucleic acids and thus a relative measure for the DNA and RNA contents.

RESULTS AND DISCUSSION

Neoblasts were discerned by their narrow cytoplasmic rim appearing red, whereas the green fluorescence of nuclear DNA superimposed on the red light emitted by nuclear and overlying cytoplasmic RNA, resulting in a bright yellow. Neoblasts from both *Dugesia tahitiensis* and *Macrostomum* n. sp. (Fig. 1) showed the same staining patterns. Controls with the classical azure A – eosin B stain (PEDERSEN, 1959) resulted in similar pictures, with RNA stained intensely blue, whereas DNA was not stained under the conditions applied. Different cell sizes, distributions and intensities of red and green colour could be distinguished visually (Fig. 2) and characterized by quantitative video microscopy. Various staining patterns were obtained from somatic cells, either differentiated or in the course of differentiation (Fig. 3). These cells differed in their appearance from neoblasts. As they approached a spherical form upon isolation, they could not be classified with certainty.

A pilot study proved the same staining protocol to be applicable to macerated planarian tissues (BAGUÑÀ & ROMERO, 1981). Cells retained their characteristic shape in

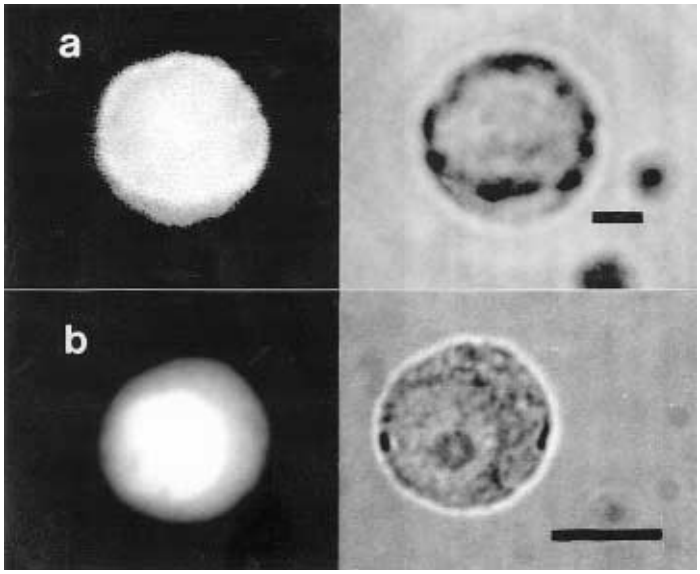

Fig. 1. – Neoblasts from a) *Dugesia tahitiensis*, b) *Macrostomum* n. sp., stained with acridine orange. The same cell is represented by its fluorescent image (left) and viewed with phase contrast optics at the same magnification (right); nuclear and cell borders in the phase contrast images coincide with the respective delineations seen through the fluorescence microscope. Scale bars: 3 µm.

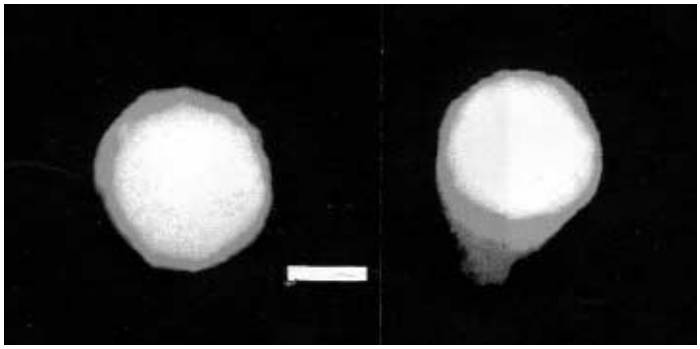

Fig. 2. – Two neoblasts from *Dugesia tahitiensis* differing in nucleocytoplasmic ratio, stained with acridine orange. The dark rim corresponds to the red area in the coloured image. Scale bar: 5 µm.

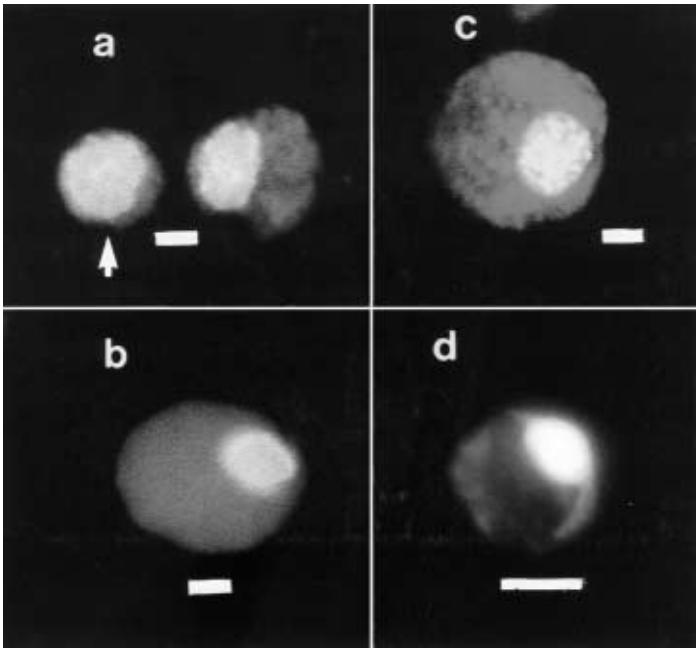

Fig 3. – Selected differentiation stages of turbellarian cells stained with acridine orange:
a) large neoblast (arrow) and differentiating cell of *Dugesia tahitiensis*;
b) differentiating cell of *Dugesia tahitiensis*, possibly similar to the "striped cell" described by BAGUÑÀ & ROMERO (1981);
c) differentiated cell of *Dugesia tahitiensis*, d) differentiated cell of *Macrostomum* n. sp. Figs (c) and (d) may represent gland or parenchymal cells.
Without characters other than size and staining pattern, all these classifications must remain tentative at present. Scale bars: 5 µm.

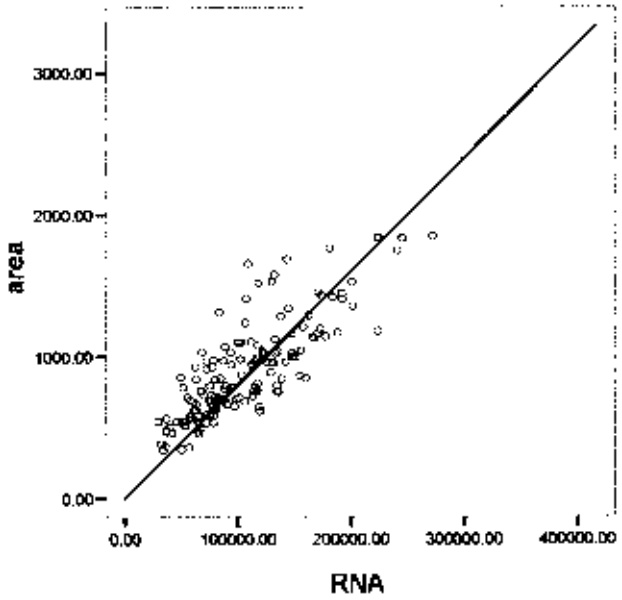

Fig. 5. – Scatter plot with linear regression of RNA contents of neoblasts from *Dugesia tahitiensis* (same disintegration as for Fig. 4) given in units analogous to those for DNA and calculated in a similar way versus total areas of cells in pixels (representing total cell volume).

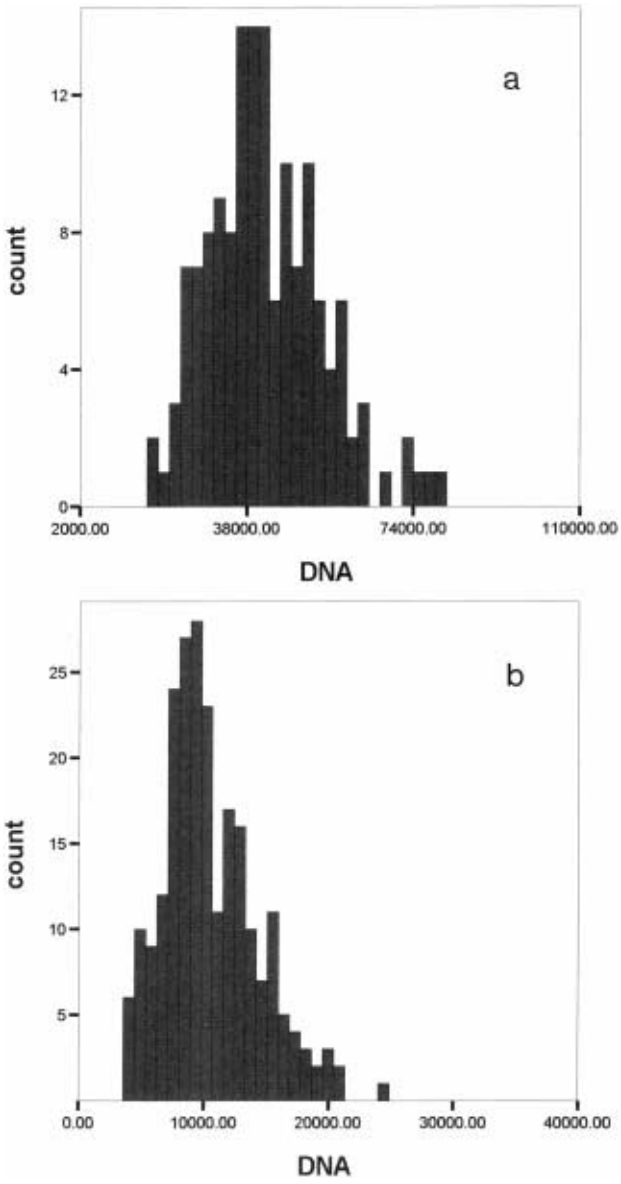

Fig. 4. – Histograms representing distributions of DNA contents for neoblasts of
a) *Dugesia tahitiensis* and b) *Macrostomum* n. sp. Abscissae: total green fluorescence as a measure for DNA contents, expressed in relative units calculated by multiplying the mean green values with the respective area in pixels. 5 specimens of *Dugesia* and approximately 100 individuals of *Macrostomum* were disintegrated and processed to yield these histograms.

this case. This may, in future, serve as a bridge for a reliable classification of cells different from neoblasts, solely on the basis of size and staining pattern. As gradient centrifugation requires intact cell membranes, the respective protocol was, however, not applied further in the present study.

First studies showed similar distributions of DNA contents (Fig. 4) for *Dugesia tahitiensis* and for *Macrostomum*, the latter with a lower DNA content, in accordance with the smaller neoblast size. These distributions met the expectations for proliferating cells: as cells in G1-phase contain half the DNA amount of G2 and mitotic cells, with those in S-phase ranging in between, a continuous distribution of DNA contents over a twofold range should be found. In fact, this range was somewhat larger. These findings are in agreement with related results for lymphocytes (see, for example, Watson, 1991) and may be explained by the influence that the accessibility of DNA exerts on staining intensity. This accessibility depends, in turn, on the cell cycle phase. Interestingly, RNA contents appear to increase proportionally with cell size (Fig. 5). The image analysis program package allows us to produce classifications based on fluorescence intensities and morphometric parameters, and to indicate fluorescence intensities in profiles drawn through cells.

Some caution in fine tuning the staining conditions has to be observed when analyzing cells different from neoblasts, as other polyanions may bind to acridine orange. Especially lysosomes may accumulate this dye (Darzynkiewicz & Kapuscinski, 1990). By constructing plots and histograms based on the analytical results, neoblast populations may be characterized and cellular dynamics monitored, as for example during regeneration and asexual reproduction of *Dugesia tahitiensis* or during growth and regeneration in *Macrostomum*. As differentiated cells possess a DNA amount corresponding to the G1 phase and are larger than neoblasts, they should easily be discriminated by image analysis. In a similar way, various lymphocyte populations have been monitored by flow cytometry (Watson, 1991: 246-259).

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