CYTO CHECK

Cytostatic Drug Testing Kit for Flow-Cytometry

part I: intracellular pH determination

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CYTOSTATIC DRUG TESTING ON PATIENT TUMOR CELLS

Explanation of the method and description of the test procedure

1. Determination of intracellular pH and esterase activity in vital cells by flow-cytometry

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1.1 DCH and ADB dyes

2,3-dicyanohydrochinon (DCH, MW 160) is a fluorescent pH indicator dye (1). The dye changes the colour of fluorescence from blue to green in the pH range 5.0 to 9.0. DCH is particularly useful for flow cytometric studies because first: only one light source is needed for fluorescence excitation, and second: because the intracellular pH determination depends on a fluorescence ratio and not on a fluorescence intensity as with fluorescein (2) or umbelliferone (3) dyes. Intensity changes in living cells are sometimes difficult to interpret. This becomes clear if one considers that fluorescent pH-indicators are brought as esterase substrates into the cell. The intensity of the stain is, therefore, primarily a measure of esterase activity. Intensity changes can only be interpreted as pH changes when changes of esterase activity, cell membrane permeability or cell volume are excluded. The advantage of a fluorescence ratio measurement is that the result is independent of the fluorescence intensity within the range of measurable fluorescences.

The incubation of vital cells with DCH in a physiological medium (e. g. 0.15M NaCl, 10 mM TRIS/HCL pH 7.35, (TBS)) shows black dots on a blue fluorescent background under the microscope i. e. the dye is excluded by the cells. Esterification with acetate (Fig. 1a) (1,4-diacetoxy-2,3-dicyano-benzene, ADB, MW 244), butyrate, or an alanyl derivative yields substances which easily permeate the cell membrane (4). The ester molecules are cleaved in vital cells within a few minutes at room temperature by cytoplasmic esterases and the resulting DCH enriches in the cells. The principle of action is similar to the permeation and intracellular cleavage of fluorescein-diacetate by cytoplasmic esterases, followed by the accumulation of free fluorescein in vital cells (5).

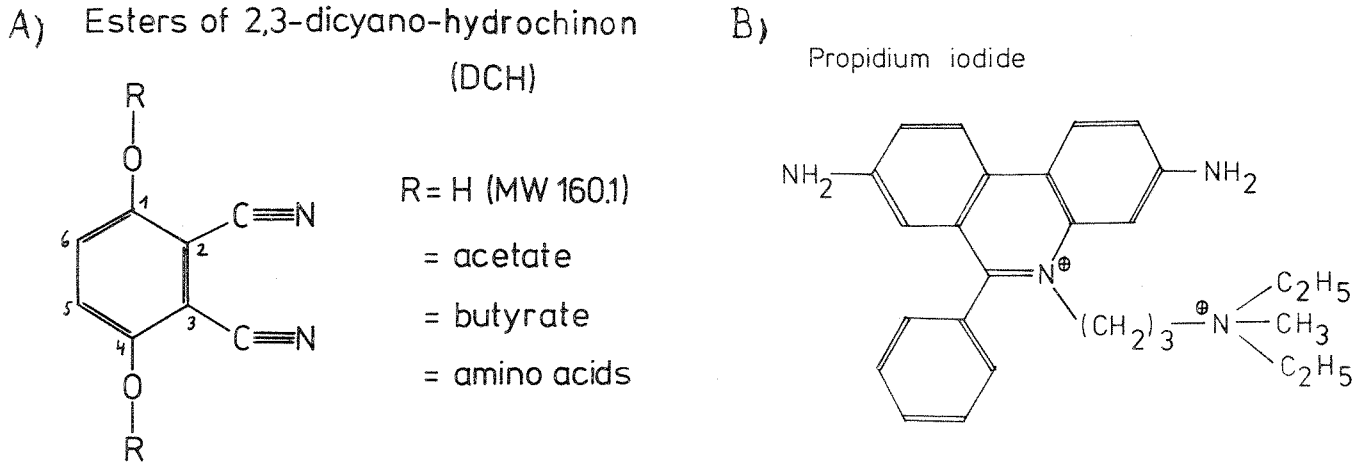


Fig. 1 DCH-Ester (A) and propidium iodide (B)

1.2 Fluorescence spectra

The DCH emission spectra extend from 400 to 550 nm (Fig. 2 a), and the excitation spectra from 300 to 450 nm (Fig. 2 b). The peak of DCH light emission shifts between pH 5 and pH 9 from 445 to 474 nm. ADB is similarly excited as DCH but emits only half of the fluorescence intensity. The peak of ADB light emission remains at 432 nm and is independent of the pH value of the solution. ADB is, therefore, in a strict sense not a fluorogenic substrate as e. g. fluorescein-diacetate which is non fluorescent as an ester. The influence of uncleaved ADB on the results of an intracellular pH-measurement with DCH is, however, small, first: because of the lower molar fluorescence of ADB and second: because of the lack of ADB enrichment in cells. DCH which is cleaved from ADB by intracellular esterases, in contrast, is strongly enriched in vital cells but does not or only weakly accumulate in dead cells.

The fluorescence excitation and emission spectra of propidium iodide (Fig. 1 b) are shown in Fig. 2 c. PI stains the DNA of dead cells but does not stain vital cells. ADB and PI are spectroscopically and chemically compatible dyes. It is, therefore, possible to determine simultaneously the intracellular pH and the esterase activity of vital cells, and the DNA distribution curve of dead cells in fresh cell preparations with a staining cocktail containing ADB and PI.

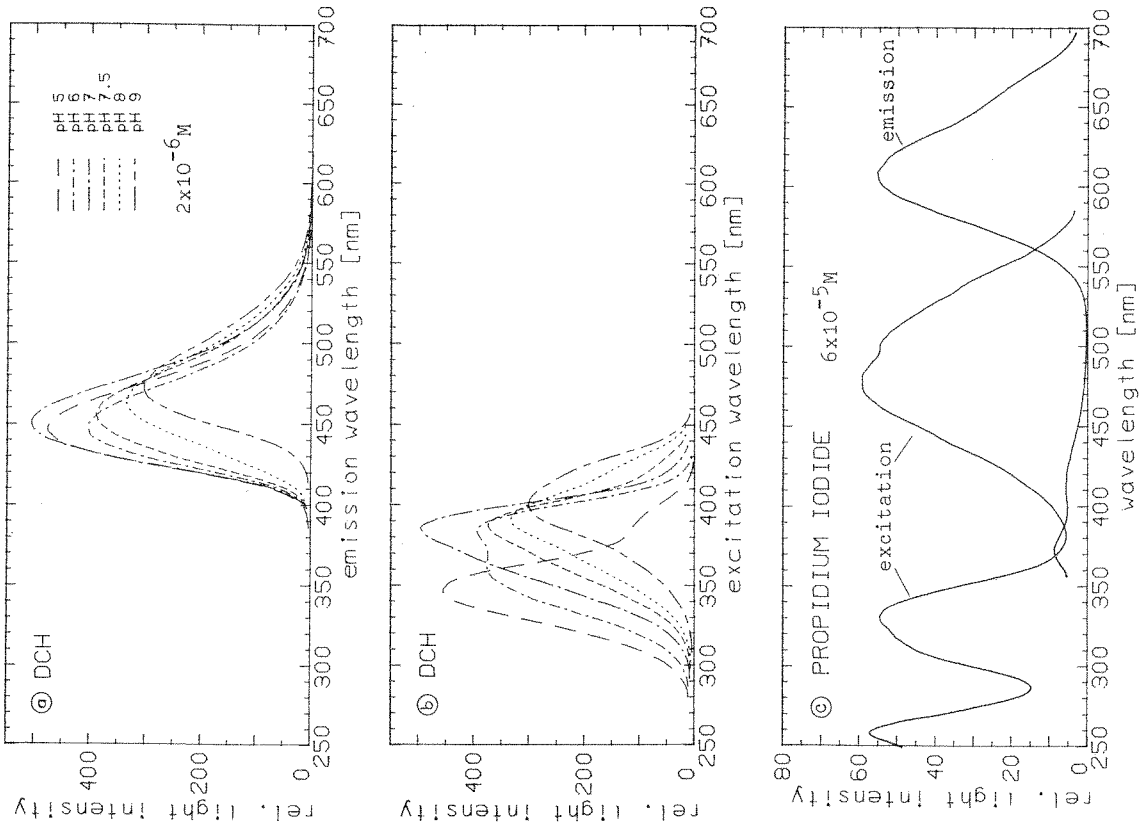
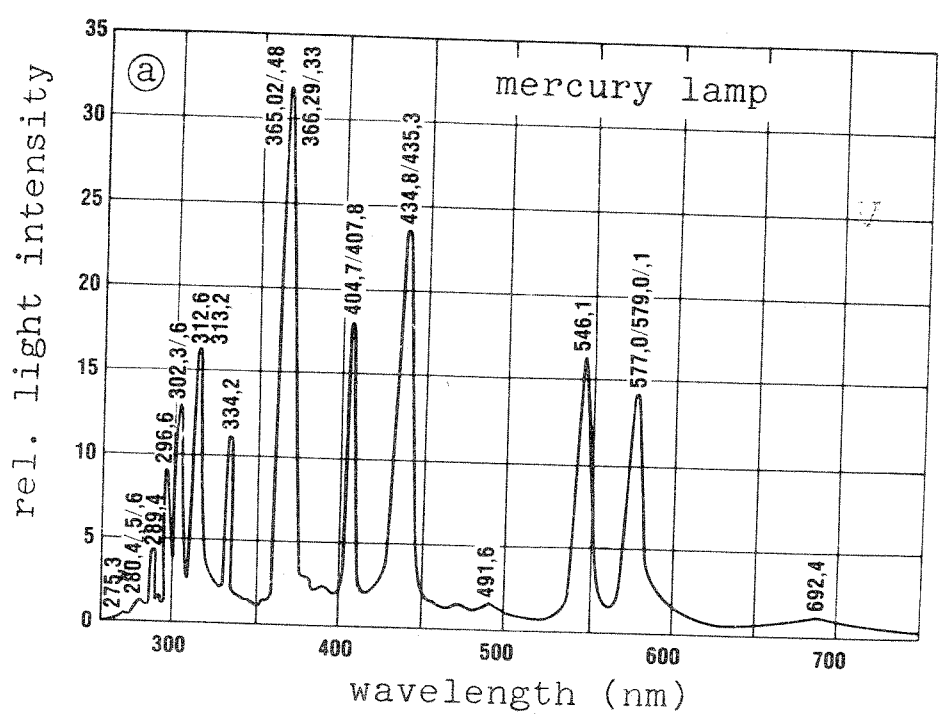


Fig. 2 Emission (a) and excitation (b) spectra of DCH in 0.15 M NaCl, 50 mM PO_4 buffer pH 5 to 9, and of propidium iodide (c) in TBS. The emission spectra were measured by setting the excitation monochromator of the fluorimeter (Perkin-Elmer-LS 5, Überlingen, FRG) to the peak wavelength of the excitation spectrum at each pH-value. The excitation spectra were obtained by setting the emission monochromator to the peak wavelength of the emission spectrum at each pH-value.

1.3 Fluorescence excitation

The fluorescence of DCH and propidium iodide can be excited between 300 and 400 nm with a HBO 100 mercury arc lamp (Fig. 3 a) or with the UV-lines of an argon or krypton laser (Fig. 3 b, 3 c).



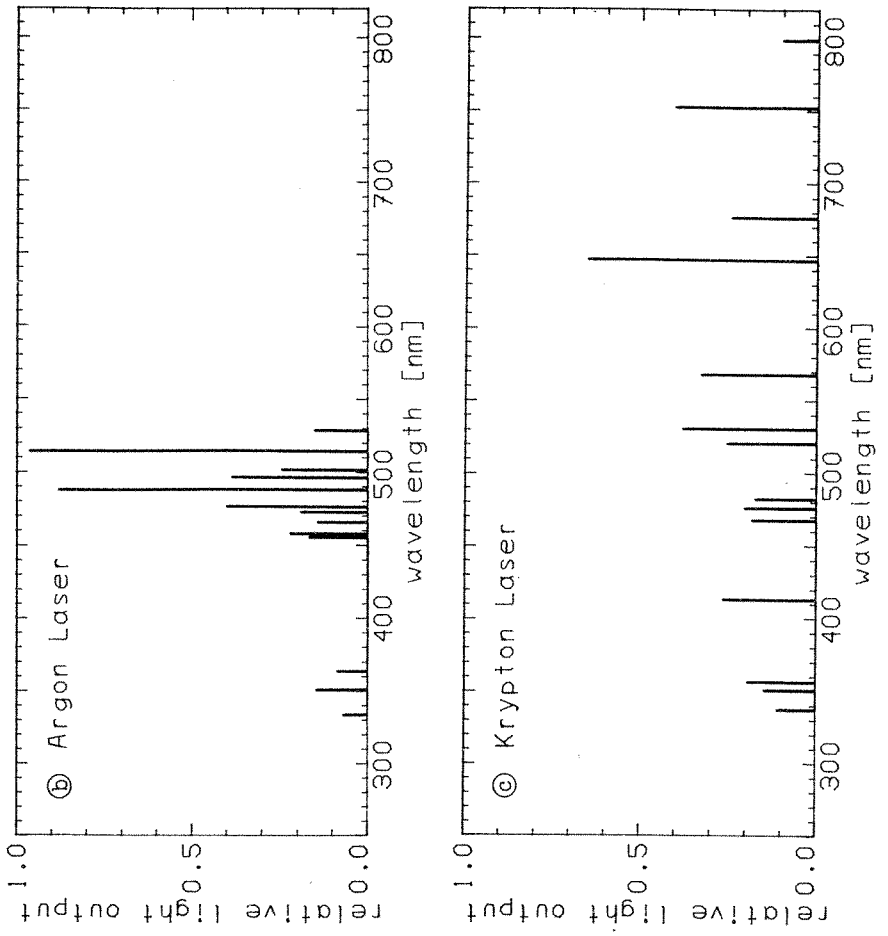


Fig. 3 Light emission spectra of a HBO-100 mercury arc lamp (a) (adapted from Leitz, Wetzlar, FRG), an argon (b) and a krypton (c) laser (adapted from Spectra-Physics, Darmstadt, FRG).

1.4 ADB assay with cells

1.4.1 Material provided

1 ml staining cocktail containing 1 mg/ml ADB and 2 mg/ml PI,
1 ml standard particle suspension, 1×10^7 particles/ml (60 μm^3, 4.88 μm diameter).

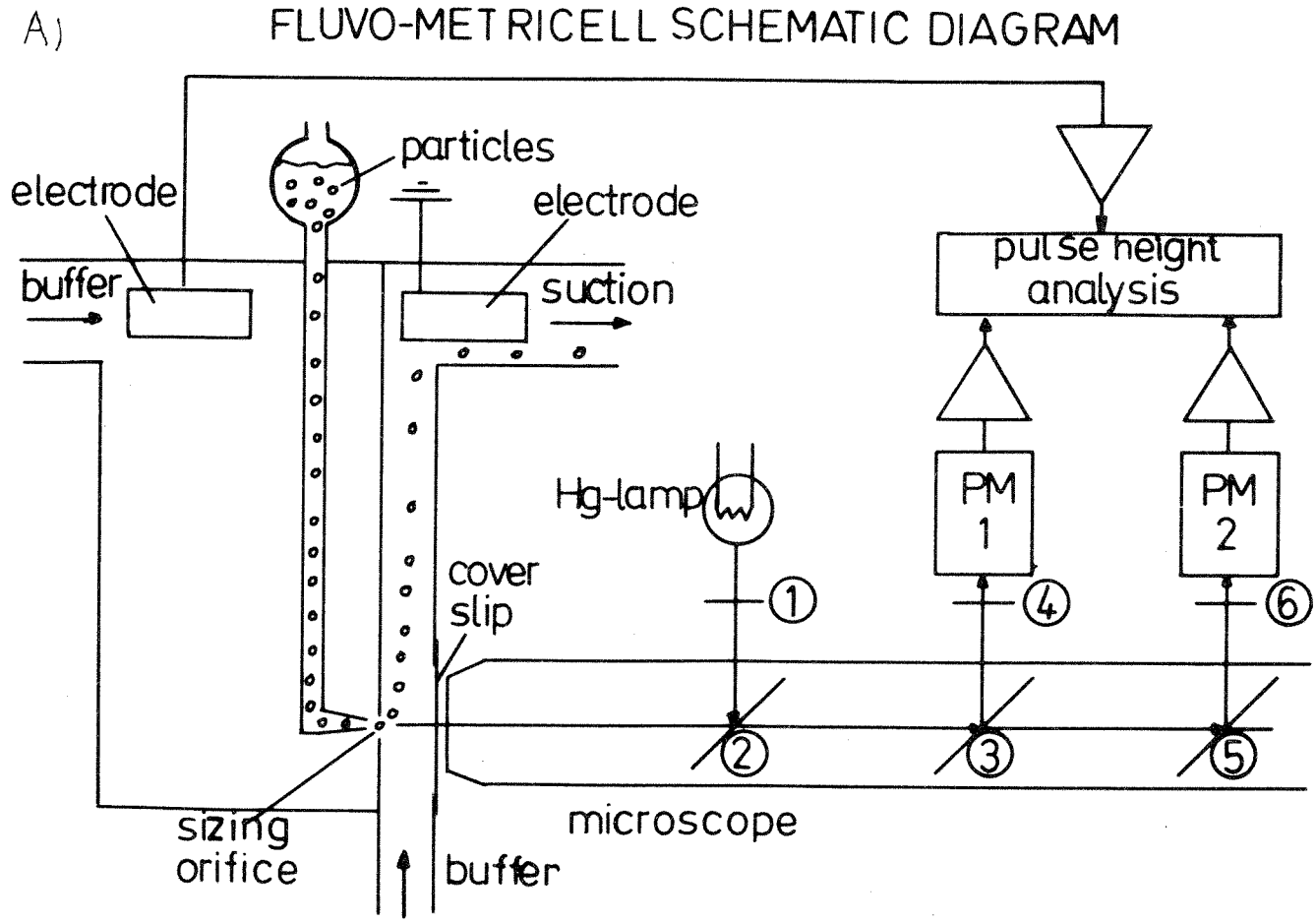
1.4.2 Assay procedure

250 μl cell suspension (1×10^6 to 1×10^7/ml) are incubated with 5 μl of a staining cocktail containing 1 mg/ml ADB and 2 mg/ml PI. The solvent of the cocktail is dimethyl-formamide (DMF). 5 μl of monodisperse, 2,3-dicyanohydrochinon (DCH) stained latex particles (1×10^7/ml, 60 μm^3, 4.88 μm diameter) as internal standard for cell concentration, cell volume and cell fluorescence are also added. The advantage of DCH-particles is that the extracellular pH of the solution can be monitored together with the intracellular pH in the same measurement.

If two parameter measurements are performed, the cells are either stained with ADB (cell volume versus esterase), with PI (cell volume versus DNA) or with ADB/PI cocktail (esterase versus DNA). DCH particles are added for standardization. The staining time for all assays is 5 min at room temperature.

1.5 Flow-cytometric measurement

The assay is typically measured in a flow cytometer which determines simultaneously the cell volume by an electrical measurement and two cellular fluorescences (Fig. 4 a). The cell volume as measured in an orifice with a diameter between 60 and 100 μm and a 1 : 1 diameter to length ratio. The orifice current is set between 0.2 and 0.5 mA. The fluorescence is excited with a HBO-100 high pressure mercury arc lamp. It is also possible to use flow cytometers or sorters with lasers, provided the laser has UV-capability in the 300 to 400 nm range. The small angle forward light scatter is measured in this case instead of the electrical cell volume.



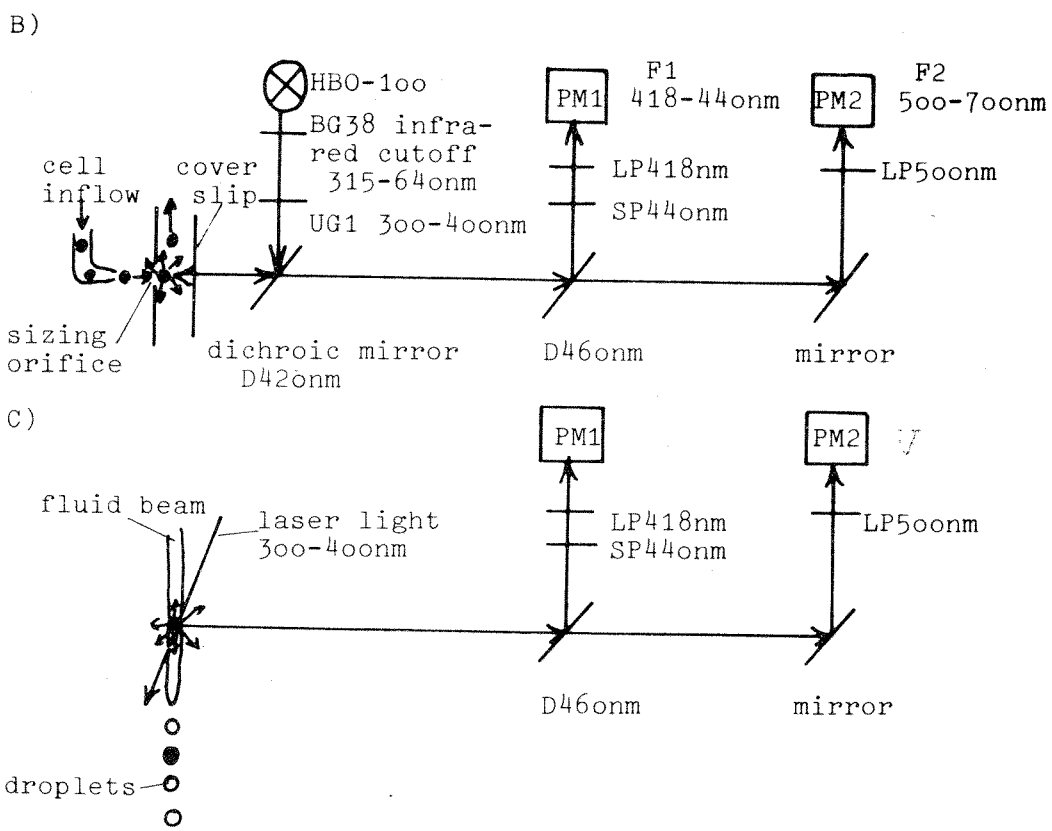


Fig. 4 Schematic diagram of the FLUVO-METRICELL flow cytometer (6) (A) with filters (B), and filter settings for laser excitation (C). PM = photomultiplier, LP = long pass filter, SP = short pass filter, F 1 = blue fluorescence light channel (PM 1), F 2 = green + red fluorescence light channel (PM 2), ① to ⑥ are filters and dichroic mirrors explained in (B) and (C).

The assay can also be measured as a sequence of two parameter histograms (see data evaluation), in case the flow-cytometer or sorter measures only the small angle light scatter and one fluorescence.

1.6 Light-pass in the flow-cytometer

Fig. 4 b indicates the filters and dichroic mirrors (all Zeiss, Oberkochem, FRG) of the FLUVO-METRICELL flow cytometer (6–8) (Fig. 4 a) which was used for the development of this assay. Also indicated is the possibility to use UV-light from an argon or krypton laser for fluorescence excitation (Fig. 4 c).

ADB and PI are both excited between 300 and 400 nm. The blue fluorescence of DCH is collected between 418 and 440 nm (F 1) and the green fluorescence of DCH between 500 and 700 nm (F 2). The red fluorescence of PI is also collected between 500 and 700 nm.

1.7 Signal processing

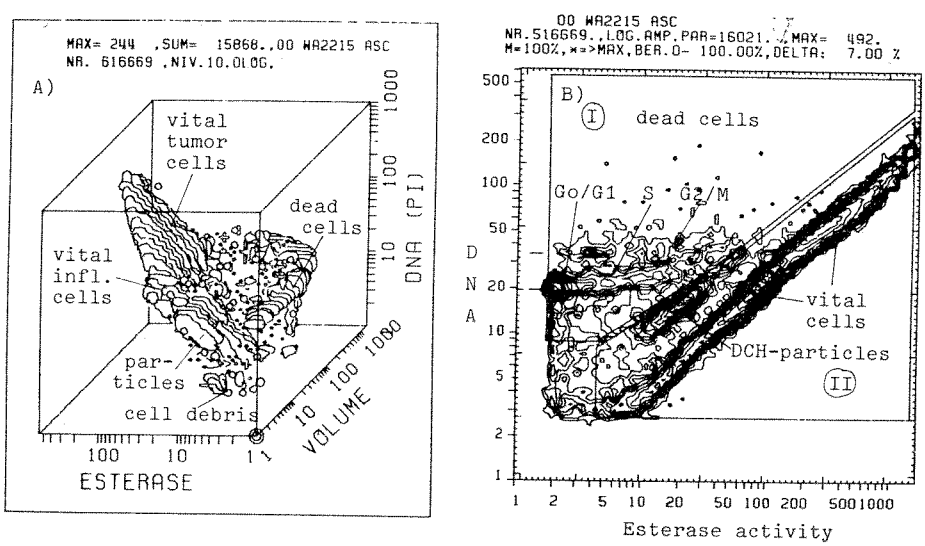
The maximum amplitude of the volume and the two fluorescence signals of each cell are amplified by 2.5 or 3 decade logarithmic amplifiers. The digitized pulses are collected in LIST-MODE on magnetic tape or disc, or classified ON-LINE in the 64 x 64 x 64 or 32 x 32 x 32 matrix in the memory of a computer and stored permanently on tape or disc at the end of the measurement.

Two parameter histograms are measured, in case no facility for three parameter measurements is available. They are stored in a multichannel analyzer for later analysis with a computer, or they are collected ON-LINE in the memory of a computer and stored on tape or disc as above.

A new and comparatively cheap possibility of measuring and analyzing two parameter histograms without need for a computer is given by the CYTOMIC microprocessor data systems (7, 8) (HEKA, D-6701 Forst/Weinstrasse, FRG).

1.8 Data evaluation

The three parameter matrix is graphically displayed as a cube (Fig. 5 a, optional) for three parameter measurements. This is useful for a quick qualitative visual evaluation. The quantitative calculations is started by projecting the data of the cube onto the esterase/DNA (blue versus yellow fluorescence) plane (Fig. 5 b). The dead (I) and vital (II) cell are distinguishable. The area (II) is used as a gating area to display the cell volume versus esterase activity histogram of the vital cells (Fig. 5 c). The absolute concentration of the cells in the various clusters of Fig. 5 c can be calculated since the one logarithmic volume class corresponds to 13 μm^3. The fluorescence scales are in relative units. The amplitude comprises 3 logarithmic



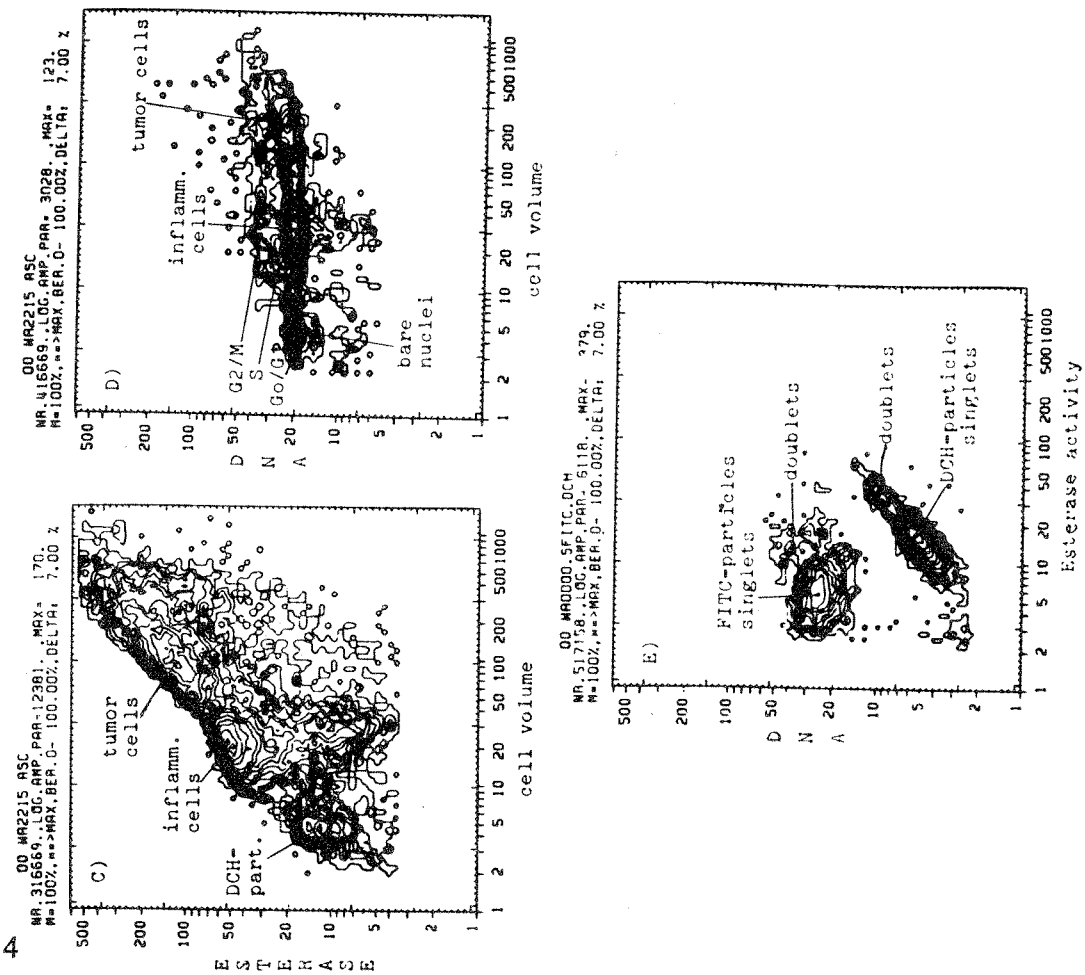


Fig. 5 Three parameter cube display (A) of an ADB/PI stained ascites of an ovarian cancer. The area (II) of the esterase/DNA histogram (B) is used as gating area for the display of the cell volume/esterase histogram of the vital cells (C), and area (I) to gate the cell volume/DNA histogram of the dead cells (D). The position of the clusters of a mixture of FITC or DCH stained calibration particles (E) in the esterase/DNA histogram frame indicates that DCH particles fall within the area of vital cells and FITC particles in the area of dead cells (compare with B).

decades. The logarithm of all channel contents is calculated and the maximum content is set as 100 %. Contour lines are drawn in steps of 7 % downward. The lowest contour line contours channels with a content of one cell.

Concentration of the particles in the assay is known (2 x 10^5/ml). The volume versus DNA histogram of the dead cells (Fig. 5 d) is obtained by using the gating window (I) of Fig. 5 b for the calculation. Fig. 5 e shows the localisation of the FITC-particles and DCH-calibration particles in the esterase/DNA histogram.

The following evaluations of the three parameter cube matrix are used to extract a maximum of information on the vital and dead cells of each measurement. The distribution of the esterase concentration (Fig. 6 a) is calculated by dividing the esterase activity (Fig. 5 c) of each histogram channel by the cell volume of each channel. The intracellular pH distribution (Fig. 6 b) is calculated from the ratio of blue to green fluorescence (F 1/F 2) of each vital cell according to the calibration curve of Fig. 7. The DNA distribution curve of the dead cells (Fig. 6 c) is calculated as projection of Fig. 5 d onto the DNA-axis. Similarly the cell volume distribution curves of vital and dead cells (Fig. 6 d, 6 e) are obtained by projection of Fig. 5 c and Fig. 5 d onto cell volume axis.

If the instrument does not collect three parameter data, one can first measure the cell volume versus esterase activity of vital cells of a sample stained with ADB alone (similar to Fig. 5 c) and in a second measurement determine the blue (F 1) versus green (F 2) fluorescence of the same sample in order to calculate the F 1/F 2 ratios of the cells. The F 1/F 2 ratio is a measure of the intracellular pH (Fig. 7). The cell volume versus DNA histogram (similar to Fig. 5 d) of the dead cells can be determined in an aliquot of the sample by staining the cells with PI alone.

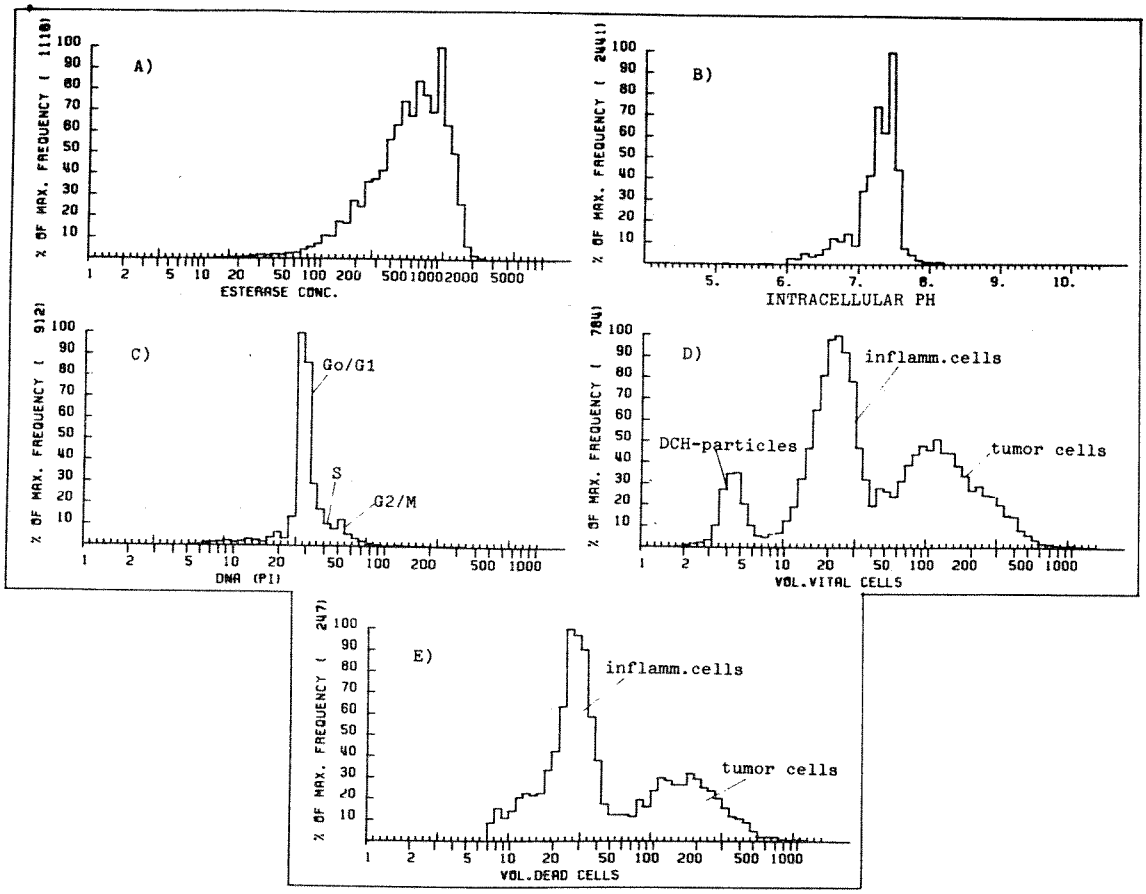


Fig. 6 One parameter distributions calculated from the simultaneous three parameter measurement of Fig. 5 a.

1.9 Calibration of pH-measurement

The calibration of the intracellular pH-measurement is made in two steps:

1.9.1 Instrument calibration

The first step concerns the calibration of the spectral response of the flow cytometer. A 0.15 M NaCl solution is buffered with 50 mM PO_4 and individual buffer samples are prepared with pH-values between pH 5.0 and 9.0 in steps of 0.5 pH units. 250 μl of each buffer are mixed with 5 μl of a DCH solution 1 mg/ml in DMF and introduced into the instrument similarly as a cell sample. Two digital voltmeters are clamped to the analog output of the two photomultiplier tubes of the flow-cytometer. The voltage readings of the photomultipliers prior to the sample input are taken as blank values. Once the fluorescence of the DCH containing sample beam is visible, the readings of the voltmeter increase. The voltage of the blue fluorescence photomultiplier (F 1) and of the green (F 2) fluorescence photomultiplier are measured three times for each buffer. The blank values are subtracted from the readings and the F 1/F 2 ratio of each measurement is calculated. The F 1/F 2 ratios determined experimentally for all 9 buffers in flow-cytometer are then plotted versus the pH of the buffers (Fig. 7). The graph is different for different flow cytometers because other photomultipliers and minor changes in the specification of optical filters and dichroic mirrors influence the result. Once established, the F 1/F 2 versus pH plot represents the permanent calibration curve of the particular flow-cytometer. The calibration curve, according to our experience, is very stable. Spontaneous and repeated measurements with our FLUVO-METRICELL flow cytometer during a period of 3 years yielded virtually superimposable curves, provided no changes of amplifier gain, photomultipliers and filters were made.

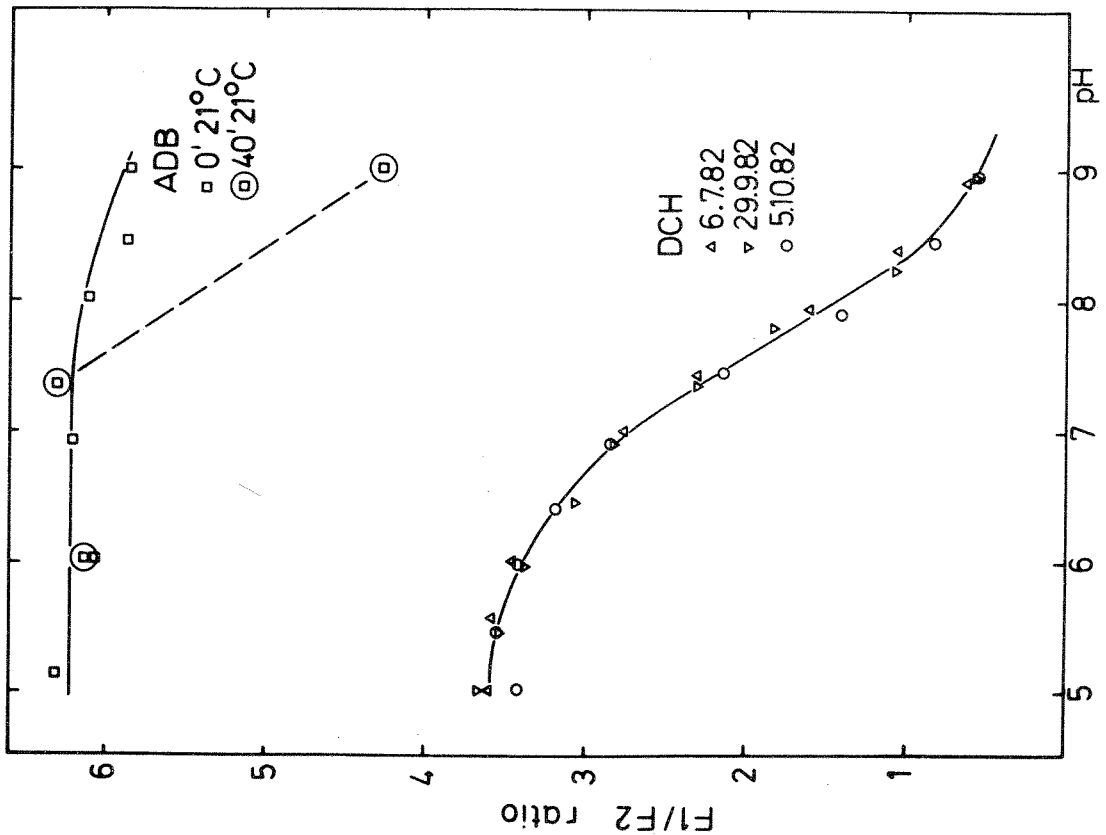


Fig. 7 Blue (F 1) to green (F 2) fluorescence ratio of a DCH and ADB solution measured in a FLUVO-METRICELL (6) flow cytometer. The DCH curve is S-shaped and pH-dependent while the F 1/F 2 ratio of ADB ratio is essentially pH-independent. Prolonged incubation at pH 9.0 leads to partial ADB hydrolysis which decreases the F 1/F 2 ratio.

1.9.2 Cellular calibration

The second step of the calibration procedure consists of the extension of the instrument calibration curve of Fig. 7 to a cellular level. The sequence of the cellular pH-calibration is first: to load the cells with DCH, and second: to expose them to buffers of various pH values in the presence of the H^+-ionophor gramicidin-D and of Na-azide. The H^+-ionophor clamps the intracellular pH to the extracellular pH and the Na-azide inhibits cellular counter regulations by poisoning the cellular energy metabolism.

Assay procedure

250 μl cells (1 x 10^7/ml) are incubated with 5μl ADB 1 mg/ml in DMF for 5 min at room temperature to load the cells with DCH. 10 μ aliquots of the DCH-stained cells are then mixed into 250 μl of buffers of various pH. 5 μl gramicidin-D (2 mg/ml in TBS) solution and 5 μl of a 1 M Na-azide solution are added to each pH-assay. After 20 min incubation at room temperature the blue to green fluorescence histogram of the cells (F 1/F 2) is measured, and the mean F 1/F 2 ratios of all cells is calculated.

Results

The results of a cellular calibration experiment are shown in Fig. 8. It is visible that incubation of the cells with gramicidin-D alone leads between pH 6.5 and 7.5 to a similar calibration curve as the DCH-calibration curve of the flow-cytometer. The curve deviates, however, below and above these pH values. Poisoning of the energy metabolism with Na-azide adapts the lower part of the curve better to the DCH-calibration curve of the flow-cytometer.

The upper part of the curve between pH 7.5 and pH 8.5 remains higher than the DCH-curve. Whether the intracellular pH in this range does not adapt to alkaline

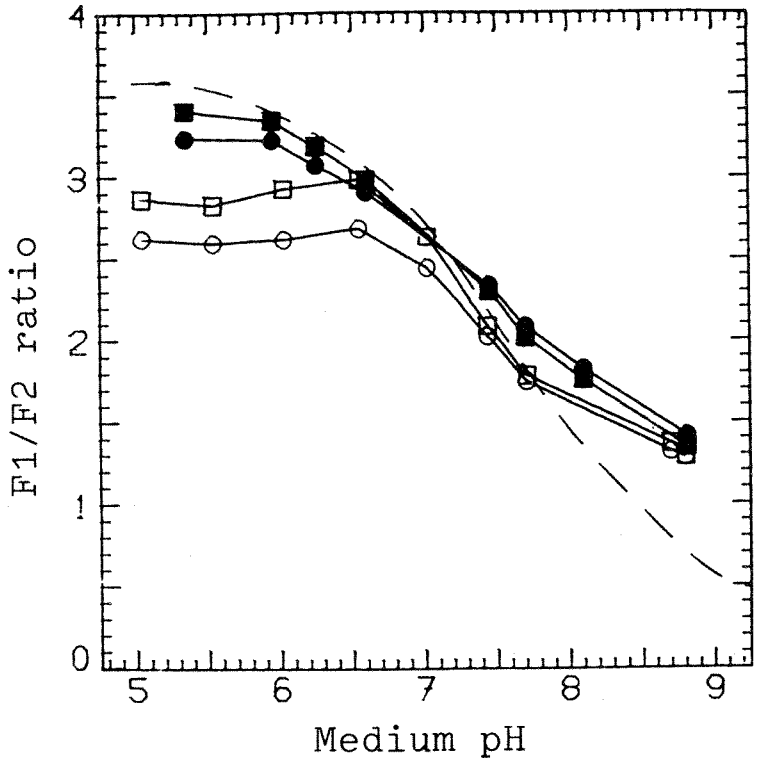


Fig. 8 Intracelullar pH calibration of guinea pig macrophages (□, ■) and granulocytes (○, ●) of the peritoneal cavity. The cells were incubated with the H^+-ionophor gramicidin-D (40 μg/ml (□, ○) and with gramidicin-D plus Na-azide (20 mM) (■, ●). A cellular calibration curve is obtained in the pH-range 5.5 to 7.5 with gramicidin-D and Na-azide which closely resembles to the dashed DCH curve measured in solution in the flow-cytometer (Fig. 7).

conditions, or whether a particular behaviour of DCH in the cell in this pH range occurs, is not clear. In practice, it is recommended to use the DCH-calibration curve of the flow-cytometer for the calculation of the intracellular pH. This is easier than establishing each time a cellular calibration curve. The approach is reasonable because the DCH-calibration curve of the flow-cytometer and cellular pH-calibration curve coincide well in the pH range 5.5 to 7.5 which is the most important for physiological regulations. Above pH 7.5 the trend of intracellular changes is still correctly indicated but the absolute pH-values calculated from the DCH-calibration curve of the instrument may be slightly lower than the true intracellular pH-values.

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