Measurement of intracellular pH in cultured cells by flow cytometry with BCECF–AM

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Abstract

This study evaluates the suitability of flow cytometry with the fluorochrome BCECF for measuring the intracellular pH (pHi) of cultured cells, and monitors the changes in pHi in murine hybridoma in batch culture and chick embryo fibroblast in monolayer culture (5th passage). The technique produced highly reproducible, repeatable results. The theoretical sensitivity from the calibration curve was 0.0004 pH units. But analysis of the standard deviation of the histogram of the green/red fluorescence ratios indicated a mean sensitivity of 0.08 (0.07–0.09) pH units. Interference due to cell size, fluorochrome incorporation and esterases were minimized by establishing a calibration curve with the cells whose pHi was to be measured using the 525/610 nm fluorescence ratio after excitation at 488 nm. The pHi of exponentially growing, batch cultured hybridomas was 7.50 at the start of culture. pHi increased during the exponential growth phase and dropped towards cell death. The pHi of the chick fibroblasts in monolayer culture was 7.30.

Keywords: Intracellular pH; Flow cytometry; BCECF; Hybridoma cells

1. Introduction

The intracellular pH (pHi) appears to be closely involved in the regulation of many metabolic pathways, including glycolysis and gluconeogenesis, and its maintenance within narrow limits is fundamental to cell viability, as it provides the appropriate environment for the wide range of intracellular activities.

Several methods have been used to measure pHi, including the distribution of weak acids and bases (Waddell and Butler, 1959), 31 P-NMR spectrometry (Inone and Yoshioka, 1989), microelectrodes (De Hemptiune, 1980) and fluorimetry with pH-sensitive probes (Bassnett et al., 1990; Van Adelsberg et al., 1989). All these methods provide information on the average intracellular pH of a population of cells. Even though microelectrodes can measure the pH of individual cells, only a few cells can actually be measured.
Flow cytometry was first developed for fundamental biology of cells and medicine with cell DNA content analysis and lymphocytes immunophenotyping. This technology analyses cells on the basis of single cell parameters but on a large population (Shapiro, 1988). The application of flow cytometry to biotechnology (Degelau et al., 1992; Alrubeai et al., 1991) is recent and is of interest because optimization of biotechnological processes requires detailed insights into the biological system involved. This technique provides detailed information on the distribution of cell characteristics and so contributes to the understanding of the physiology of the cultured cells.

Three types of fluorochrome are presently available for determining pH by flow cytometry. One is ADB (1,4-diacetoxy 2,3-dicyanobenzene) which is hydrolysed to DCH (2-dicyanohydroxyquinone) and requires UV excitation. DCH diffuses out of cells rapidly (Cook and Fox, 1988). The second is SNARF1 (carboxyseminaphthorhodafluor I), the newest fluorochrome for this application (Justement et al., 1990; Van Erp et al., 1991; Wieder et al., 1993). It is somewhat difficult to use because it suffers from rapid photo-bleaching (Bassnett et al., 1990). The third is BCECF (2',7'-bis-carboxyethyl-5,6-carboxyfluorescein), which is excited by visible light (488 nm) and diffuses out of cells very slowly. Its ease of use also makes it the most suitable fluorochrome for standardizing pH measurements by flow cytometry.

Several studies have been carried out on a variety of cells (Hedley and Jorgensen, 1989; Neubauer et al., 1989; Tron et al., 1989; Van Erp et al., 1991) and on hybridomas (McQueen and Bailey, 1991) to determine the function of pH in cell processes and its relationship with other metabolic indicators. The present study evaluates the technique of measuring pH by flow cytometry using BCECF (Musgrove et al., 1986; Musgrove and Hedley, 1990) on murine hybridoma cells and chick embryo fibroblasts. The linearity, reproducibility, fluorochrome leakage and the conditions under which the fluorochrome provided reliable results were all determined. The system was then used to measure pH in cells to assess measurement accuracy on cells grown in a bioreactor.

2. Materials and methods

2.1. Cell cultures

2.1.1. Hybridoma cells: support independent cells

Murine hybridoma (CRL 8001, ECACC) cells, producing IgG specific for CD3 antigens, were grown in RPMI 1640 supplemented with 8 mM glutamine and 10% fetal calf serum (FCS) in a 5% CO2 incubator at 37°C.

The batch cultures in the bioreactor were run in 2 l tanks (Biolaffite, France) with a working volume of 1.2 l. The inoculum was obtained from exponentially growing cultures maintained in 75 ml T flasks at a density of 3.10^5 cells per ml. The medium pH was kept at pH 7.0 by automatic injections of 1 M NaOH or CO2. Two samples were removed each day for determining cell count, cell volume (Coulter counter), cell viability (by trypan blue exclusion and flow cytometry from two-parameter histograms FALS X 90LS), and intracellular pH by flow cytometry. The cell suspension is used directly for labelling.

2.1.2. Chick embryo fibroblasts: support dependant cells

The pelvic cartilage of 11-day-old chick embryos was dissected out in minimal essential medium (MEM) containing 5% non-essential amino acid mixture (Flow Labs), 5% 200 mM gentamycin, 20 μg ml^-1 streptomycin and 100 μg ml^-1 penicillin. The pieces of cartilage were then digested with 0.25% trypsin-0.2 mg ml^-1 collagenase in MEM for 2 h at 37°C with gentle agitation. The supernatant containing the fibroblasts was centrifuged for 10 min at 900 g at 30°C. The cell pellet was resuspended in MEM containing 10% FCS and the cell density determined with a haemocytometer. A volume of 10 ml containing about 1 x 10^4 cells per ml was placed in 75 cm² Falcon flasks and incubated at 37°C in a non-humidified atmosphere of 5% CO2.

Confluent cells were removed by incubation with 0.025% trypsin in phosphate-buffered saline (PBS) for 10 min. at 37°C, collected by centrifugation at 900 g for 10 min at 30°C and sub-cultured. Fibroblasts in their 5th passage were used in all studies. The cells used for pH determination were labelled immediately after trypsinization.
2.2. Reagents

A 1 mM stock solution of BCECF-AM (Molecular probes, Interchim, Montluçon, France) in dimethyl sulfoxide was prepared and stored at 4°C. Earl’s balanced salt solution (EBSS) was 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, 5 mM glucose and 25 mM HEPES. The pH was brought to 7.3 ± 0.1 with 1 M NaOH or Tris and checked with a PHN 75 pH meter (Tacussel Electronique, Villeurbanne, France) at room temperature (precision 0.01 pH units). A set of high [K+] buffers with pH of 6.0–8.0 in 0.2 pH unit steps was prepared by mixing appropriate proportions of (135 mM KH₂PO₄, 20 mM NaCl) and (110 mM K₂HPO₄, 20 mM NaCl) at room temperature. All buffers were stored at 4°C. A stock solution of nigericin (Sigma), 1 mM in absolute ethanol was kept at 4°C.

2.3. Cell labelling

The cells suspension was washed rapidly once with EBSS buffer pH 7.3 and resuspended in the same solution at a final concentration of 10⁷ cells per ml. BCECF-AM was then added to a final concentration of 10 μM and the suspension incubated for 25 min at 37°C to allow BCECF-AM to be hydrolysed by intracellular esterase into pH-dependent fluorescent BCECF.

Optic fluorescence microscope controls shown that these are optimal conditions of labelling with 99% of the cells being labelled and labelling intensities allowing good voltage and photomultiplier gain setting of the cytometer. Then aliquots of 10⁶ cells were collected, centrifuged at 250 g and stored on ice until resuspension in 1 to 2 ml of fresh EBSS immediately before cytometric analysis.

The calibration curve was constructed from labelled cells resuspended in high [K+] buffer of appropriate known pH, containing 10 μM nigericin for 5 min before flow cytometry. Calibration curves (fluorescence ratio against pH) were established for each experiment using cells from the same whose pH₁ has to be measured.

2.4. Control of cell integrity during labelling

We have checked that the labelling procedure does not affect the pH₁ of the cells:

First, washed cells were incubated at 20°C during 0.5, 1, 1.5, 2, 3, 4 and 5 h before the labelling step and then the pH₁ was determined.

Second, labelled cells were incubated at 37°C during 0.5, 1, 1.5, 2, 3 and 4 h and then the pH₁ and the viability were determined.

Third, a cell suspension was divided in two aliquots. One was treated as described above, the second was labelled directly in the culture flask, centrifuged and analyzed by fluorometry.

A calibration curve was realised for each aliquot.

2.5. Flow cytometry

Cells were analyzed in an Epics C Coulter flow cytometer (Coultronics, Margency, France) equipped with a Coherent argon laser (Innova 90.5) with an excitation wavelength of 488 nm and a 200 mW power. The green fluorescence (F1) was selected using a 525 nm bandpass filter, while the red fluorescence was obtained with a 610 nm long-pass filter. The ratio of the two signals was used in all experiments. It was calculated directly by the cytometer data analysis system and presented as a 1024-channel histogram (Fig. 1). The ratio is given by the mean peak channel of the histogram ratio after analysis of 25000 cells. The optical alignment was checked each day, all other setting were unchanged throughout the study.

3. Results

3.1. Control of cells labelling

The washed cells incubated at 20°C for variable time before labelling step have a pH₁ which remains stable during 1.5 h at a value of 7.6. Then the pH₁ decreases to a value of 7.35.

![Fig. 1. Green (525 nm) and red (620 nm) fluorescence histograms and green/red fluorescence ratios of cells labelled with 10 μM BCECF and resuspended in EBSS.](image-url)
Loaded cells incubated for variable time at 37°C have also a pH$_i$ of 7.6. Decreasing pH$_i$ (up to a value of 7.30 after three h) is observed after one h of incubation, concurrently with decreasing viability.

The observed pH$_i$ were 7.48 and 7.46, respectively, for cells directly labelled in the culture flasks and cells loaded with the procedure described above.

### 3.2. Calibration curves: linearity and sensitivity

The samples were analyzed in a different order in each experiment to avoid any variation due to any excess dye release or excess dye uptake (Kolber et al., 1988; Prosperi, 1990). The channel of the mean peak ratio gradually increased as a function of pH$_i$ (in equilibrium with pH$_e$) from 6.0 to 8.0 (Fig. 2). This displacement of the mean peak ratio as a function of pH can be given as a calibration curve (Fig. 3).

#### 3.2.1. Linearity

Calibration curves were prepared for each of the 8 cell samples, taken after different times in culture. All the curves had the same shape and were essentially parallel. A normalization test on the 8 curves showed that all points were perfectly superimposable (Fig. 4A, B). There exists a shift of the curves towards higher fluorescence of the cells up to 32.5 h, the intensity then gradually dropped until the fluorescence at 80 h was below that of cells taken at zero h. The calibration curve was linear over the range pH 6.6–7.4 (regression coefficient, $r = 0.99$), the curve flattened above pH 7.4. The linear regression coefficient over the range 6.6–7.6 was $r = 0.98$. The correlation coefficient over the range 6.6–7.6 was $r = 1.00$ using a third order polynomial curve fit.

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**Fig. 2.** Shift of the ratio histogram with pH. The 525/620 fluorescence ratio histograms are for OKT3 cells labelled with 10 μM BCECF and resuspended in high [K$^+$] buffers containing nigericin.

**Fig. 3.** Calibration curve: mean peak ratio plotted against pH$_i$. The calibration curves were constructed from the mean peak ratio histogram as a function of pH.

**Fig. 4.** (A) 8 calibration curves: Cell samples were taken twice a day for 4 consecutive days from a culture growing in a bioreactor (4A). (B) the same curves after normalization.
Table 1

| pH<sub>1</sub> values obtained by three types of calculation |
|-------------------|-------------------|-------------------|
| **Time (h)**      | Sample            | pH 6.6–7.4<sup>a</sup> | pH 6.6–7.6<sup>b</sup> | pH 6.6–7.6<sup>c</sup> |
| 0                 |                   | 7.38 ± 0.09         | 7.47 ± 0.15           | 7.54 |
| 8.5               |                   | 7.40 ± 0.09         | 7.47 ± 0.13           | 7.52 |
| 22.5              |                   | 7.42 ± 0.09         | 7.51 ± 0.13           | > 7.60 |
| 32.5              |                   | 7.50 ± 0.07         | 7.58 ± 0.10           | > 7.60 |
| 47                |                   | 7.47 ± 0.07         | 7.56 ± 0.11           | > 7.60 |
| 56.5              |                   | 7.37 ± 0.07         | 7.43 ± 0.11           | 7.42 |
| 70.5              |                   | 7.33 ± 0.08         | 7.40 ± 0.12           | 7.37 |
| 80                |                   | 7.36 ± 0.08         | 7.41 ± 0.11           | 7.38 |

<sup>a</sup> Linear regression for a calibration range of 6.6–7.4.
<sup>b</sup> Linear regression for a calibration range of 6.6–7.6.
<sup>c</sup> Polynomial regression for a calibration range of 6.6–7.6.

3.2.2. Sensitivity

The theoretical sensitivity (obtained from the displacement of the mean peak ratio channel) was 0.004 pH units. The sensitivity of the technique was also estimated from the standard deviation of the ratio histogram. The mean standard deviation of the histogram ratio was 18 channels, so that the sensitivity was 0.08 pH units over the range 6.6–7.4. The values in Table 1 show the pH<sub>1</sub> of cells determined after various times in culture using three types of calibration curve. They confirm the sensitivity, showing that the mean standard deviation for pH determination was 0.07 pH units.

3.3. Reproducibility and resolution

The repeatability and reproducibility of the technique were determined. Triplicate samples prepared in high [K<sup>+</sup>+] buffer at pH 6.4, 7.0 and 7.6 were analyzed. The experiment was repeated three consecutive days (Table 2). The coefficients of variation (CV) for the measurements made 3 days apart were below 3% at each pH tested, and the CV was < 1% for measurements made on the same day. The reproducibility was confirmed by including two samples containing nigericin, one at 7.15 and the other at 7.25, but not used for the calibration curve, in each assay. The measured values were 7.18 ± 0.02 for the 7.15 sample and 7.25 ± 0.02 for the 7.25 sample (the values calculated from a linear regression over the range 6.6–7.4 each day are shown in Table 3). The accuracy of the technique is due to the high resolution, as indicated by the CV below 5% for the histogram ratio.

3.4. Dye leakage

The release of fluorochrome before and after adding nigericin was examined by analysing the green fluorescence histogram. The pellets of labelled

Table 2

<table>
<thead>
<tr>
<th>Samples *</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>CV inter assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.6</td>
<td>819&lt;sup&gt;b&lt;/sup&gt;</td>
<td>883</td>
<td>828</td>
<td>826</td>
</tr>
<tr>
<td></td>
<td>CV = 0.7%</td>
<td>CV = 0.4%</td>
<td>CV = 0.2%</td>
<td>CV = 0.6%</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>748</td>
<td>758</td>
<td>751</td>
<td>744</td>
</tr>
<tr>
<td></td>
<td>CV = 0.8%</td>
<td>CV = 0.8%</td>
<td>CV = 0.6%</td>
<td>CV = 1.6%</td>
</tr>
<tr>
<td>pH 6.4</td>
<td>649</td>
<td>636</td>
<td>640</td>
<td>631</td>
</tr>
<tr>
<td></td>
<td>CV = 1.2%</td>
<td>CV = 0.08%</td>
<td>CV = 1.6%</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Linear regression for a calibration range of 6.6–7.4.
<sup>b</sup> Linear regression for a calibration range of 6.6–7.6.
<sup>c</sup> Polynomial regression for a calibration range of 6.6–7.6.
cells were kept on ice before adding nigericin. An aliquot was then analyzed immediately and the others at various later times. There was no significant loss of fluorescence before 120 min. Similarly, the fluorescence of labelled cells resuspended on EBSS (pH 7.2) and kept on ice remained stable for 2 h (Fig. 5). The fluorescence of labelled cells resuspended in high [K+] buffer containing nigericin appeared to be less stable over time, and about 10% fluorescence was lost after 2 h.

### 3.5. The pH<sub>i</sub> of hybridoma cells and fibroblasts

Table 1 shows the pH<sub>i</sub> of OKT3 cells grown in a bioreactor and sampled after different times in culture. The values were obtained using three types of calculation: linear regression over the ranges 6.6–7.4 and 6.6–7.6, and polynomial regression over the range 6.6–7.6. The pH<sub>i</sub> determined by all three calculation models was the same. It indicated that the intracellular pH increased during the growth phase and became more acidic at the beginning of cell death (Fig. 5). The absolute value of the pH<sub>i</sub> was difficult to determine, however, the average pH<sub>i</sub> of the OKT3 cells in growing phase was 7.50, and that for fibroblasts was 7.3.

Among other determined parameters, the one of the hybridoma cells in culture that changed first was the cell volume (measured in the Coulter counter), it had changed by the 32<sup>nd</sup> h in culture (Fig. 6). Cell viability, estimated by trypan blue exclusion or cytometry (FALS X 90LS), decreased significantly only after 47 h (the two curves were similar but cytometry gave lower values).

### 4. Discussion

This study evaluates and standardizes measurement of pH<sub>i</sub> by flow cytometry using BCECF. One of the difficulties in cytometry is the lack of a standard methodology, and this makes interpretation of results difficult. We have also tested the suitability of flow cytometry as a measuring technique by examining the precision (repeatability, reproducibility), linearity and sensitivity.
4.1. Selection of BCECF

Although several fluorochromes are presently available for measuring $pH_i$ by flow cytometry, BCECF is the most widely used (Hedley and Jorgensen, 1989; McQueen and Bailey, 1991; Musgrove and Hedley, 1990; Musgrove et al., 1986; Wang et al., 1990) for two main reasons. First, because it is excited by visible light, and second, because it is released very slowly from labelled cells (the fluorescence of cells kept on ice is stable for at least 2 h). Our initial trials with this fluorochrome on lymphocytes and fibroblasts indicated satisfactory linearity and sensitivity and showed that the method was practicable. The method was then optimized in subsequent trials on cells in culture. This provided large numbers of readily accessible cells that were already in suspension for repeated measurements. DCH was not used because it is released from cells very rapidly, and must be excited by UV light (Cook and Fox, 1988). Lastly, SNARF has not yet been widely used enough and its behaviour is not completely documented (Bassnett et al., 1990; Wieder et al., 1993).

4.2. Use of the fluorescence emission ratio

All measurements of $pH_i$ were made using the ratio of the fluorescence emitted at 525 and 620 nm (green/red). This ratio has been shown by several groups (Hedley and Jorgensen, 1989; Musgrove et al., 1986) to correct for changes in cell volume and fluorochrome uptake within a single sample of cells. While the 620 nm fluorescence is not really an isosbestic point, it is true that the fluorescence is not $pH$ dependent at this wavelength, so providing for some correction. The use of this ratio provided increased sensitivity, as the CV for the histogram ratios were < 5%.

4.3. Measurement evaluation

The different assays achieved to control cell integrity during labelling show that cells loss their own capacity to control actively their $pH_i$ after several incubation hours. Our procedure (needing 20 min incubation) seems to be available to be used for bioprocess monitoring and does not affect the intracellular $pH$. The intra-assay repeatability of measurements is very good; it was below 3% for measurements made on different days and better than 1% for same-day measurements. The reproducibility was also satisfactory. Normalization tests (Fig. 4B) showed that the calibration curves prepared on different days were completely reproducible. The displacement of curves towards higher fluorescence is probably due to changes in intracellular esterase activity. This discrepancy was maximal at 32.5 h, at which time cell metabolism is maximal, and then gradually decreased. The change in cell volume cannot be responsible for the increased fluorescence, as the cell volume is maximal at 22.5 h in culture.

The sensitivity estimated under these assay conditions was 0.08 $pH$ units, which is similar to those obtained by others (Musgrove et al., 1986; Wang et al., 1990). This sensitivity seems to be lower than that obtained with DCH (Cook and Fox, 1988) or SNARF (Van Erp et al., 1991; Wieder et al., 1993), but the calculation methods were not standardized.

Calibration was linear over the range 6.4–7.4, and hence a simple linear regression could be used for all calculations. However, if measurements must be made on cell lines having a high $pH_i$, the calibration can include the $pH$ 7.6 point. It is then preferable to prepare the calibration curve using a polynomial regression. BCECF does not seem to be the most suitable fluorochrome to use for $pH$ above 7.6, and SNARF1 could perhaps be used as its analytical range extends towards more alkaline $pH$.

4.4. Measurement of $pH_i$

The $pH_i$ of different cells measured by this technique were in agreement with published values. The $pH_i$ for lymphocytes obtained with this technique was 7.2 as reported by (Justement et al., 1990), it was 7.3 for fibroblasts (Van Erp et al., 1991), and it was 7.6 for hybridomas (McQueen and Bailey, 1991). In our study the $pH_i$ increased during the growth phase of the cells, and the drop in $pH_i$ signalled cell death. But the $pH_i$ is not the earliest indicator of cell death. The $pH_i$ begins to drop between 32.5 and 47 h in culture, while the cell volume drops between 22.5 and 32.5 h. The cell viability curves do not follow the same time scale however. Viability, measured by the classic trypan blue exclusion technique, begins to drop between 47 and 56.5 h. The drop in cell volume
thus seems to be the first indication of cell death, that is probably of apoptotic origin. Indeed increasing cell volume and incorporation of the trypan blue are characteristic of the necrotic cellular death when apoptotic death is marked by a decreasing cell volume and trypan blue exclusion (Darzynkiewicz et al., 1992). Here, cell volume and viability estimated by morphologic cytometric parameters decrease before the viability determined by trypan blue.

5. Conclusion

The technique of flow cytometry with BCECF is a reliable, simple way of determining $\text{pH}_i$. It is essential to standardize the methodology by establishing calibration curves using the same cells as those to be studied at the same time in culture and separate curves must be constructed for each cell line using adequate intracellular concentrations of fluorochrome. The technique is sensitive enough to make it useful for $\text{pH}_i$ studies. As fluorochromes have different measurement ranges, the choice of fluorochrome is determined by the cell line studied. SNARFI may perhaps be the best fluorochrome for studying hybridoma cells with a $\text{pH}_i$ of 7.50, as its analytical range is more basic (Van Erp et al., 1991). But BCECF remains a suitable label for studies on lymphocytes and fibroblasts.

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References


