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The Relationship between Viability and Intracellular pH in the Yeast *Saccharomyces cerevisiae*

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The relationship between viability (cell proliferation activity) and intracellular pH in the yeast *Saccharomyces cerevisiae* was investigated by using cells that had been deactivated by low-temperature storage, ethanol treatment, or heat treatment. The intracellular pH was measured with a microscopic image processor or a spectrofluorophotometer. At first, the intracellular pH measurements of individual cells were compared with slide culture results by microscopic image processing. A clear correlation existed between the proliferation activity and intracellular pH. Moreover, by spectrofluorophotometry analysis, it was found that there was a relationship between the viability and intracellular pH of brewing yeast under conditions of low external pH ($n = 15$, $r = 0.960$, $P = 0.001$). This relationship was also observed in baker's yeast ($n = 13$, $r = 0.950$, $P = 0.001$). On the other hand, when the fluorescein staining method was used in these experiments, the relationship between viability and staining percentage was not observed. From these results, intracellular pH was found to be a sensitive factor for estimating yeast physiology. The possible role of cell deterioration is also discussed.

Yeasts form an important class of industrial microorganisms. Their role in the food industry over the past 100 years has led to the development of numerous techniques for the study of their viability. Examples include methods based on vital staining, cell replication, and metabolic activity. However, it has been difficult to determine subtle differences in the proliferation activity of live cells (12, 13).

Intracellular pH and the H^+ pump are thought to play an important role in yeast growth and glycolysis/gluconeogenesis. The plasma membrane ATPase, which regulates intracellular pH, is essential for yeast growth (30). The transmembrane H^+ gradient is the driving force in the uptake of nutrients. Maltose (26) and amino acids (4, 5, 25) are transported along with H^+. The transmembrane H^+ gradient is the driving force in nutrient transportation, and the plasma membrane ATPase forms the transmembrane H^+ gradient (28). In addition, intracellular pH regulates the key enzymes in glycolysis and gluconeogenesis. As the key enzymes in glycolysis and gluconeogenesis are regulated by a cascade of reactions involving cyclic AMP (cAMP)-dependent protein kinases (7, 15, 17, 32, 36), cAMP plays an important role in this regulation. In addition, it was found that cAMP is regulated by intracellular pH (18). On the other hand, intracellular pH is also thought to be a trigger for the other cell responses, including the induction of heat shock proteins (35). Thus, intracellular pH is considered to be one of important factors in yeast physiology.

Recently, intracellular pH was used for the first time to predict yeast wort fermentability (10). However, the relationship between intracellular pH and cell proliferation activity has been unclear until now. In this paper, we describe our study of this matter, which is thought to be widely important to the microbiological industry.

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MATERIALS AND METHODS

Yeast strain. The lager-brewing yeast strain *Saccharomyces cerevisiae* Kl084 from our stock collection and a commercial baker's yeast source were used in this experiment. Brewer's yeast cells were cultured at 8°C for 7 days in stand flasks containing wort (11° Plato) (1° Plato = 1 g of sugar [as sucrose] per 100 g of wort at 20°C). Baker's yeast cells, which was isolated from pizza, were cultured at 25°C for 45 h in shake flasks containing yeast extract-peptone-dextrose (YEPD).

Measurement of intracellular pH. Intracellular pH was measured with a spectrofluorophotometer by the method described by Imai et al. (10). Microscopic image processing was performed as described below.

Slide cultivation experiments. After yeast cells were cultured at 8°C for 7 days in stand flasks containing wort, they were washed with cold water. They were then stored for 6 or 21 days at 1°C. The stored cells were washed once with citrate-disodium hydrogen phosphate buffer (pH 3.0), containing 110 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$, and resuspended in the same buffer. Then 5 μM 5- and 6-carboxyfluorescein diacetate (10 mM dimethyl sulfoxide solution) was added, and the suspension was vortexed for 45 min. Cells were centrifuged (3,000 × *g* for 3 min at 1°C), resuspended in the same buffer, and kept at 1°C for 105 min under gentle shaking.

This cell suspension was mixed with slide cultivation medium (0.67% yeast nitrogen base [Difco], 3% glucose, 1% carageenan [Sigma type II]) at 30°C. The mixture was immediately allowed to set on a hemocytometer to record the cell positions before and after cultivation. The intracellular pH of individual cells was measured immediately by an image-processing technique (11), and microscopic images were also recorded at a visible wavelength. After incubation for 16 h at 20°C, microscopic images at a visible wavelength were again taken.

Determination of viability. Plate culture was carried out with malt agar (Nissui Pharmaceutical) at 25°C. Then 100 μl of cell suspension diluted with a solution containing 0.145 mM EDTA and 70 mM NaCl was spread on a plate. Vital staining with 5- and 6-carboxyfluorescein diacetate was carried out by the method of Rotman et al. (24). To compare the results of intracellular pH measurements, the same cells that were used for the intracellular pH measurement by spectrofluorophotometry were observed.

Measurement of intracellular ATP concentration. The ATP concentration was measured in a BLR-201 luminescence reader (Aloka), using the luciferin-luciferase enzyme assay. A 1-volume portion of the sample was mixed with 9 volumes of dimethyl sulfoxide by the method of Hysert et al. (9). A 2-μl volume of this extract (or standard or blank) was added to 100 μl of reaction buffer (100 mM

FIG. 1. Fluorescent staining (A) and measurement of intracellular pH (B) of the same yeast cells. Yeasts which had been stored for 1 and 21 days at 1°C were mixed, stained with 5- and 6-carboxyfluorescein, and suspended in pH 3.0 buffer. The fluorescence image (B excitation) was recorded, and the intracellular pH was measured by the methods described in Materials and Methods.

Tris, 4 mM EDTA) in a scintillation vial. Then 100 μl of the luciferin-luciferase mixture (Boehringer Mannheim) was added to this reaction mixture, and the luminescence was measured.

Materials. 5- and 6-carboxyfluorescein and its acetic ester were obtained from Molecular Probes Inc; MES [2-(*N*-morpholino)ethanesulfonic acid] and dimethyl sulfoxide (Lumisonal) were obtained from Dojindo Laboratories Co., Ltd.

RESULTS

Figure 1 shows the results of fluorescence staining and the measurement of intracellular pH under conditions of low pH for the same yeast cells by microscopic image processing. The results of the fluorescein diacetate method (3, 20, 24) suggested that all the cells were alive, because all the cells are fluorescent (Fig. 1A). The intracellular pHs of the same cells under low-pH conditions were also measured (Fig. 1B). Comparison of the two pictures indicated no relationship between the fluorescence intensity (Fig. 1A) and intracellular pH (Fig. 1B) of a cell.

The relationship between intracellular pH and cell proliferation activity was further investigated, as shown in Fig. 2. Again, the results of fluorescein staining showed no difference in cell activities (Fig. 2B). However, the intracellular pH measured before cultivation was found to be different between cells (Fig. 2C). After cultivation for 16 h at 20°C, an image of the same area was taken (Fig. 2D). When Fig. 2C and D are compared, it can be seen that the intracellular pH was high in cells which produced many daughter cells whereas cells with a low intracellular pH produced few daughter cells. Measurement of the intracellular pH of the cell was found to reflect the ability of the cell to proliferate.

To investigate the relationship between subtle differences in cell proliferation and the intracellular pH under low external pH conditions, it is not suitable to use cells completely deactivated by heat. Cells which had been deactivated by a mild low-temperature treatment (storage at 1°C for 1 to 20 days) were used in these experiments. The intracellular pH at an external pH of 3.0 and the cell viabilities as determined by the spread plate culture method and the fluorescein diacetate method were measured for each yeast sample. As shown in Fig. 3, there was a linear correlation between the intracellular pH and the number of cells which proliferate ($n = 15$, $r = 0.960$, $P = 0.001$). On the other hand, many cells that appear stained did not form a colony after being spread on malt agar (Fig. 4). There was no correlation between the number of viable cells counted by the fluorescein diacetate method and the number of colonies obtained after plating. For baker's yeast, there was also a linear correlation between the viability determined by the plate culture method and the intracellular pH at an external pH of 3.0 ($n = 13$, $r = 0.950$, $P = 0.001$) (Fig. 5).

The intracellular pH and ATP content were measured after various cell treatments (Tables 1 and 2). Over a 10-min treatment at 60°C, the ATP content of the cell decreased and intracellular pH also decreased from 6.2 to 4.7 (Table 2). In the case of ethanol treatment, the fluctuation of cell physiology could be also detected by measuring the intracellular pH. After the heat or ethanol treatment in these experiments, all of the cells were stained with 5- and 6-carboxyfluorescein diacetate.

DISCUSSION

Numerous methods of assessing yeast proliferative activity have been reported, but until now it has been extremely difficult to quantitatively determine the extent of cell deactivation during industrial usage. This is because there are often subtle differences in cell activity. This paper first pointed out that intracellular pH can be used to predict cell viability. In particular, it became clear that intracellular pH could show the subtle differences of yeast proliferation activity more sensitively than ever.

The widely used fluorescein diacetate method is based on the esterase activity and membrane integrity of the cell (31). This method is efficient in distinguishing dead from live cells (12, 13). However, as shown in Fig. 1 and 2, the extent of fluorescence does not always reflect the proliferation activity of a cell. This also means that the extent of esterase activity and membrane integrity of the cell does not always show the level of proliferation activity.

In this paper, 5- and 6-carboxyfluorescein was used as a pH indicator. This compound cannot easily enter the organelles

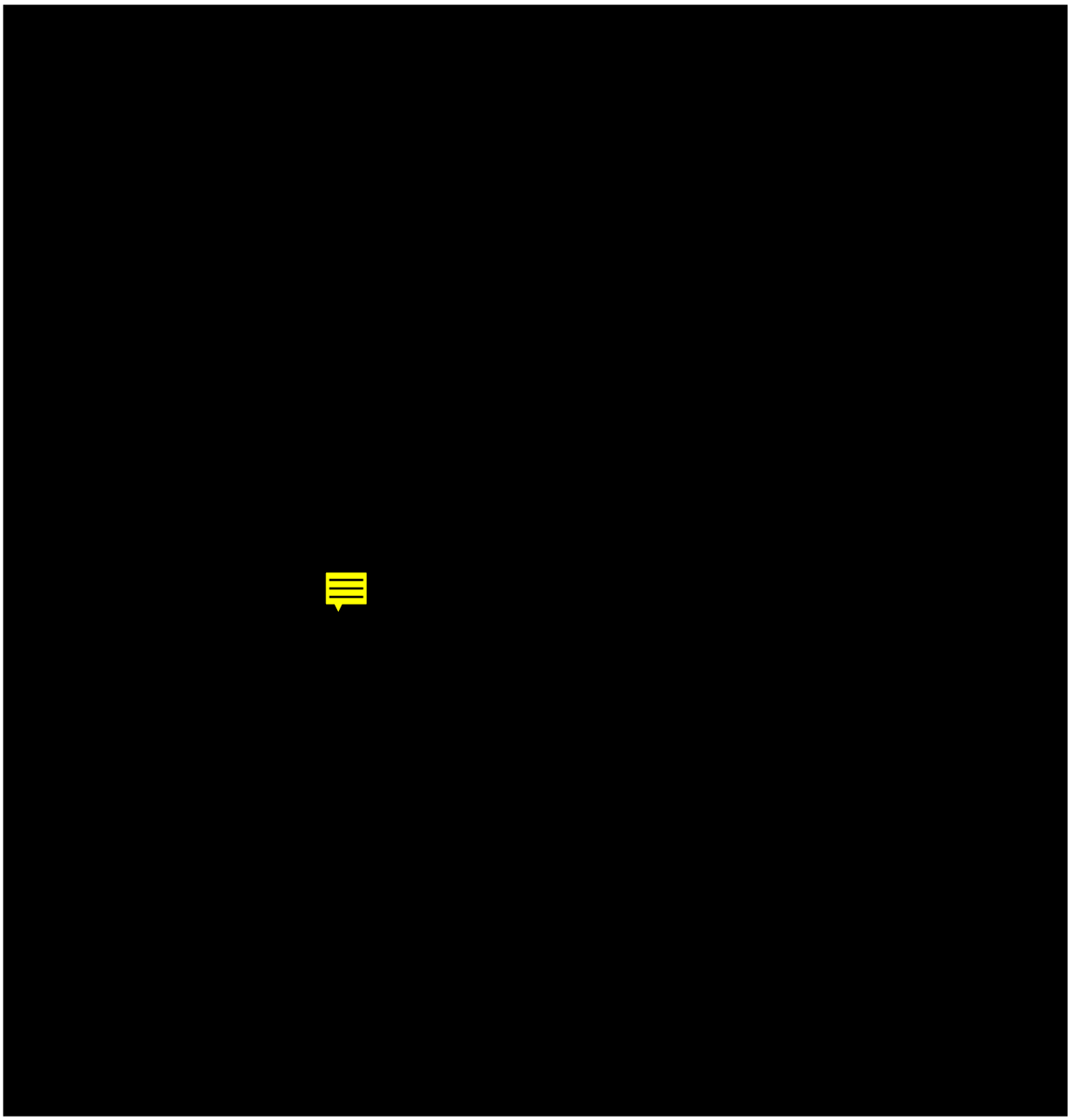

FIG. 2. Fluorescence (B and C) and intracellular pH (A and D) images of cells before (A to C) and after (D) cultivation. Slide cultivation experiments were performed as described in Materials and Methods. These images were taken from the same area of the hemocytometer. (A) Image taken with a visible wavelength before cultivation. (B) Fluorescence image taken before cultivation, using B excitation. (C) Intracellular pH image taken before cultivation. (D) Image taken with a visible wavelength after cultivation.

from cytosol (33), as also observed by fluorescence microscopy. Intracellular pH was measured under conditions of low pH (pH 3.0). (The maximum specific growth rate [reciprocal hours] of yeasts used here was constant for pH 2.8 to 6.0 [19].) As shown in Fig. 2, the proliferation activity of individual cell could be predicted quantitatively from the intracellular pH.

As shown in Fig. 3, we also found that there is a relationship between intracellular pH at external pH 3.0 and viability of cells deactivated by mild treatment, as observed during industrial usage (6, 14, 16). The same relationship was also observed for baker's yeast as well as brewer's yeast (Fig. 5). From these results, it was thought that this relationship could be observed in the yeast *S. cerevisiae*. On the other hand, it was difficult to assess difference in cell proliferation activity by the fluorescein diacetate method even if this staining was performed in the buffer without glucose at low temperature to avoid dye extrusion from the cytosol (Fig. 4).

Intracellular pH also indicated yeast cell vitality under the other deactivation conditions (either heat or ethanol treatment), and fluctuation of intracellular pH was correlated with

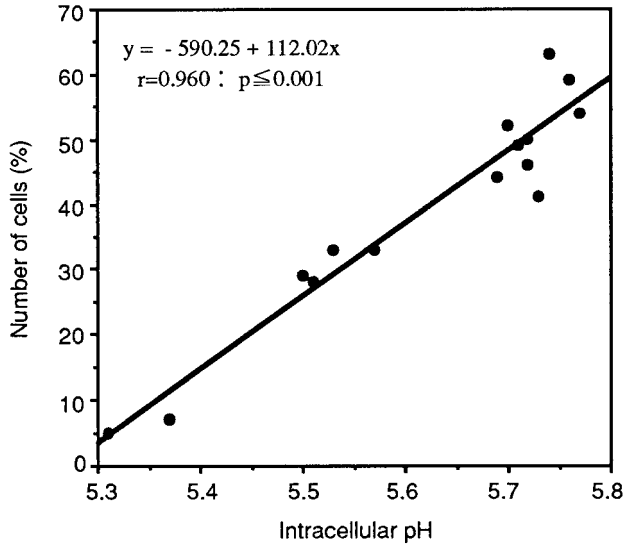

FIG. 3. Relationship between the viability of brewer's yeast cells and intracellular pH at an external pH of 3.0. Brewer's yeast cells harvested by centrifugation (3,000 × *g* for 10 min at 2°C) were washed with cold distilled water and stored at 1°C for 1 to 20 days. The intracellular pH of the cells was measured at an external pH of 3.0, and viability was determined as described in Materials and Methods.

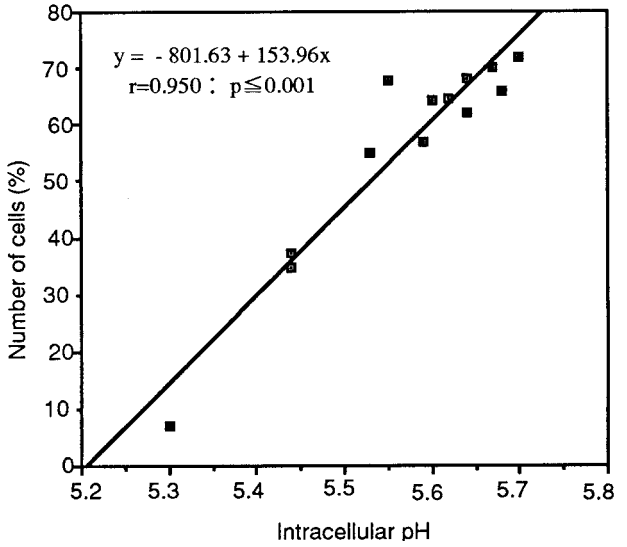

FIG. 5. Relationship between viability and intracellular pH of baker's yeast cells at an external pH of 3.0. Baker's yeast cells harvested by centrifugation (3,000 × *g* for 10 min at 2°C) were washed with cold distilled water and stored at 1 to 25°C for 1 to 16 days. The intracellular pH at an external pH of 3.0 and viability were measured as described in Materials and Methods.

a similar fluctuation of the cellular ATP content (Tables 1 and 2). Although it is not clear why there is a limitation in the fluorescein diacetate method for cells with subtle differences in vitality, the fact that there were fluorescein-stained cells with low viability in the experiment in Fig. 4 could be thought to be related in an energy-dependent manner (1).

These results show that intracellular pH under low-pH condition is a key factor to the understanding of cell physiology. The method based on intracellular pH can be applied in the microbiological industry, in which subtle differences in yeast proliferation activity are often observed, and it is expected to lead to the development of a new device for determination of yeast physiology. It will be also interesting to determine whether this principle can be applied to bacteria and mammalian cells.

Yeasts cells are thought to be prepared for death through a deactivation process (13). However, the mechanism of this deactivation process is difficult to determine. As described in this paper, there was a clear relationship between yeast intracellular pH and its proliferation activity. Yeast intracellular pH is regulated by proton-pumping plasma membrane ATPase (8, 27, 28), whose level is changed by ethanol and octanoic acids (2, 21, 23, 34). Artificial reduction of plasma membrane ATPase activity was reported to reduce growth (22, 29, 30). Therefore, it was thought that the change in proton extrusion activity is an important processes in cell deactivation, leading ultimately to cell death. The results reported here will also contribute to greater understanding of the mechanisms of cell deactivation.

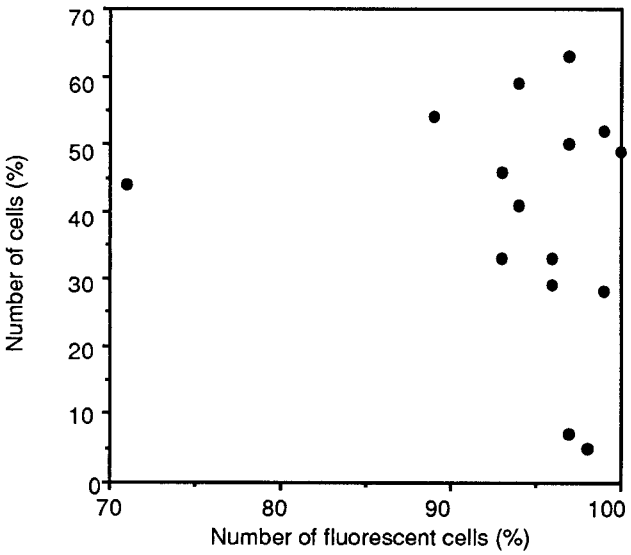

FIG. 4. Relationship between the viability of brewer's yeast cells as determined by the plate culture method and the fluorescein diacetate method. The yeast samples described in the legend to Fig. 3. were used. Viability determination was carried out as described in Materials and Methods.

TABLE 1. Effect of ethanol treatment on intracellular pH and ATP content[a]

Ethanol concn (%, vol/vol)	Intracellular pH	ATP content (μmol/g [dry wt] of yeast cells)
0	6.23	7.2
4.4	6.24	7.5
8.8	6.24	7.6
17.6	6.23	5.8
35	5.91	4.9
52	4.51	0.036
70	4.49	0.028

[a] The viability of the 0% ethanol treatment sample was 95% by slide cultivation. Yeast cells were suspended in ethanol solution at 0°C for 6 min. After centrifugation, the cells were washed twice with cold distilled water. The intracellular pH (as measured by spectrofluorophotometry) and ATP content of these samples were analyzed as described in Materials and Methods.

TABLE 2. Effect of heat treatment on intracellular pH and ATP content[a]

Heating period (min)	Intracellular pH	ATP content (μmol/g [dry wt] of yeast cells)
0	6.19	6.9
2	6.19	8.6
5	5.73	7.8
10	4.65	0.065
20	4.68	0.016

[a] Yeast cells were suspended in distilled water at 0°C in a tube. The tube was held in a 60°C water bath for the indicated time, and the cells were washed twice with cold distilled water. The intracellular pH (as measured by spectrofluorometry) and ATP content of these samples were analyzed as described in Materials and Methods.

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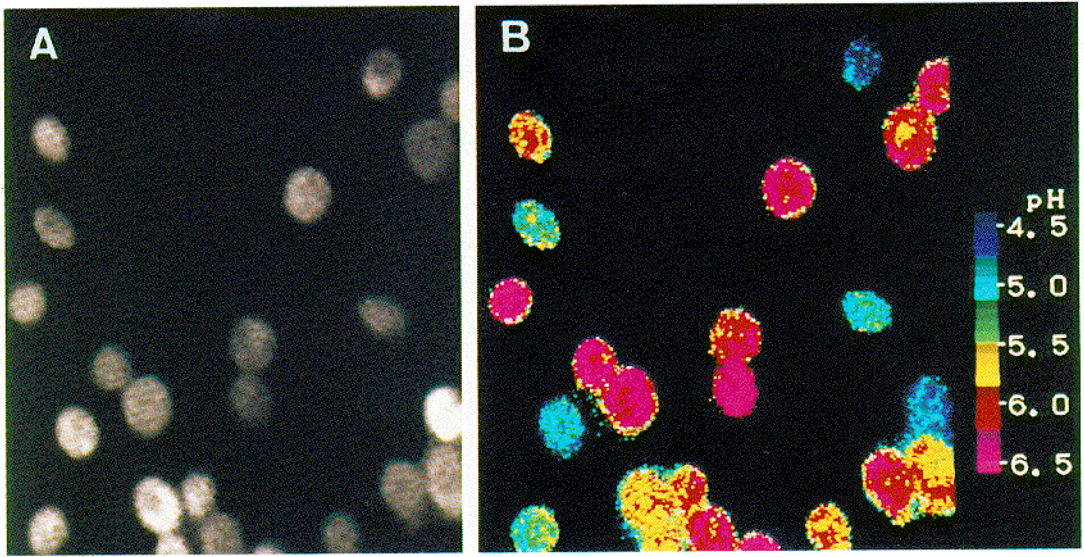
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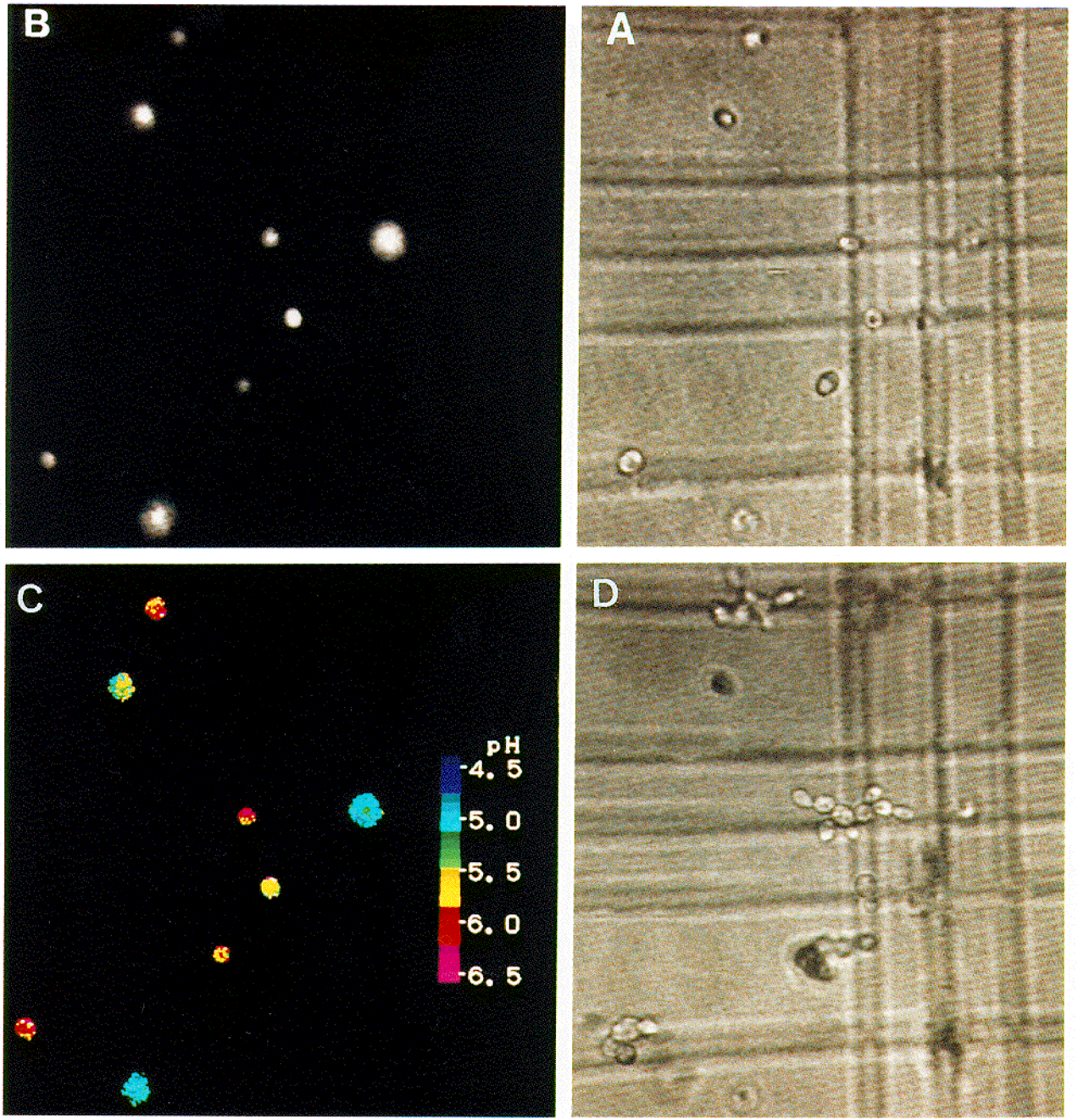
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