*Biotechnology Techniques Vol 1 No 4 213-218 (1987)*
*Received as revised November 26*

# ON-LINE MEASUREMENT OF HYBRIDOMA GROWTH BY CULTURE FLUORESCENCE

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## SUMMARY

Fluorescence measurement of viable hybridoma cell cultures provides a convenient method for monitoring the progress of a batch culture. It is shown that cell concentration changes as low as 35,000 cells/ml during initial stages of growth can be measured reliably. This sensitivity, however, decreases to 2 X $10^6$ cells/ml at cell concentration greater than 2 X $10^6$ cells/ml. The culture fluorescence of hybridoma culture is a characteristic property of the cell and the medium used. Consequently, processes in which the medium composition and cell lines are invariant, a direct on-line estimate of viable cell count can be made using the method investigated in this paper.

## INTRODUCTION

Production of large amounts of monoclonal antibodies requires a carefully developed strategy for the scale-up of existing conventional means of *in vitro* production. In bioprocesses employing hybridomas, the most important variable which influences antibody productivity is the cell concentration. Monitoring cellular growth is usually accomplished by counting the number of cells in a sample of the culture broth from the bioreactor. This approach is tedious, requires trained eyes, and is operator dependent. This paper reports on the use of a new technique for measuring hybridoma cell count using the principle of culture fluorescence.

The electron carriers NAD(P)H are present in all viable cells. The NADH and NAD(P)H play key roles in catabolism and anabolism, respectively. The cells maintain a pool of these reduced nucleotides. For example, in aerobic metabolism the NADH, produced in the citric acid cycle, is used by the respiratory chain enzymes for metabolic energy production. It is believed that under given growth conditions, the reduced nucleotide pool [NAD(P)H] per unit viable cell mass remains relatively constant. The reduced nucleotides, NAD (P) H fluoresce at 460 nm when irradiated with a 340 nm light source (Duysens & Amesz, 1957; Harrison & Chance, 1970). Hence, measurement of the emitted light of a cell culture is indicative of viable cell count in the bioreactor. It is this phenomenon that is used to monitor hybridoma growth. Although this approach has been used previously for bacterial and fungal systems (Armiger et al, 1984; Armiger et al, 1986; Beyeler et al, 1981; Luong & Carrier, 1986; Ristroph et al, 1977; Zabriskie & Humphrey, 1978), its applicability to a mammalian culture system has not been investigated. From the fluorescence measurement perspective, the mammalian cell systems

differ from the bacterial system in two significant factors. Firstly, the cell mass per unit volume in a typical reactor is lower by more than an order of magnitude. Secondly, the culture medium which is rich in amino acids and proteins, strongly absorbs the irradiation light source ( 360 nm) thus reducing the light energy reaching the cells. Both of these factors tend to reduce the sensitivity of culture fluorescence measurement. The object of this paper is to provide fundamental culture fluorescence data of hybridoma cultures.

## MATERIALS AND METHODS

The hybridoma used in this study was ATCC HB32 (14-4-4S). The cell line produces a cytotoxic monoclonal antibody ($IgG_{2a}K$) that reacts with the $I\text{-}E_k$ and $C_k$ determinants. The line was derived by fusing SP2/O-Ag14 cells with lymphocytes of C3H cells. The cited antibody production was 23 μg/ml (Ozato et al, 1980).

The medium used in the study was supplemented Dulbecco's Modified Eagles Medium (DME) with glutamine and low glucose (Hazelton/KC). The medium was supplemented with the following, added per liter of DME: 2.2g sodium bicarbonate, 10 ml nonessential amino acids (Hazelton/KC), 13.2 mg oxalacetic acid, 0.8 mg insulin, 5.5 mg pyruvic acid, 3.0 g glucose, and 110 ml fetal bovine serum (Hazelton/KC). The medium was stored at 4°C, and used within 48 h.

Inoculum was typically 600 ml of $3.3 \times 10^5$ cells/ml for 1900 ml of fresh medium (in the bioreactor) for a final cell concentration of $8 \times 10^4$ cells/ml. In all experiments, a 5-liter Techne spherical bioreactor (BR-05) equipped with a pH electrode, a dissolved oxygen electrode and a FluroMeasure® detector (BioChem Technology, Malvern, Pa) and a floating stirrer was used. The bioreactor was operated at its maximum working capacity of 2.5 liters with agitation set at 200 rpm and temperature maintained at 37±0.1°C. The head space of the bioreactor was sparged at 150 ml/min with gas of composition: 20% $0_2$, 5% $CO_2$ and 75% $N_2$ by volume.

Cell numbers were determined by the Trypan blue exclusion method using a haemocytometer. Quadruplicate cell counts were performed daily. Glucose and lactate were periodically determined by the o-toluidine colorimetric reaction (Hyvarinen & Nikkila, 1962; Henry, 1968) and lactate diagnostic kit (Sigma Chemicals), respectively. Antibody titers were determined using an enzyme-linked immunosorbent assay (ELISA) following the procedures of Bosworth et al (1983).

The FluroMeasure® System (MacBride et al, 1986) is an open-ended fluorimeter, miniaturized within a probe for insertion directly into a culture vessel through a 25 mm fitting. It contains a stable source of UV light (350±40 nm), a detector for the emitted fluorescent light (450±40 nm), and appropriate optical filters. Constant intensity of UV light is accomplished by a feed back control system, which keeps the detector in calibration for 1000 h. All experiments reported in this paper were conducted without the detector requiring re-calibration. The culture fluorescence is expressed in terms of normalized fluorescence units (NFU). One NFU unit corresponds to a change in fluorescence caused by 0.122 μM NADH at 30 °C, pH 8.0, in the range of 1 to 25 μM NADH.

## RESULTS AND DISCUSSION

Figure 1 shows typical profiles of culture fluorescence, pH and concentrations for viable cells, glucose, lactate, monoclonal antibody, and dissolved oxygen. In all batch experiments, the cell count reached a peak at about 75 h. At this time oxygen became limiting with the dissolved oxygen concentration approaching zero. The viable cell count decreased beyond 75 h. The antibody

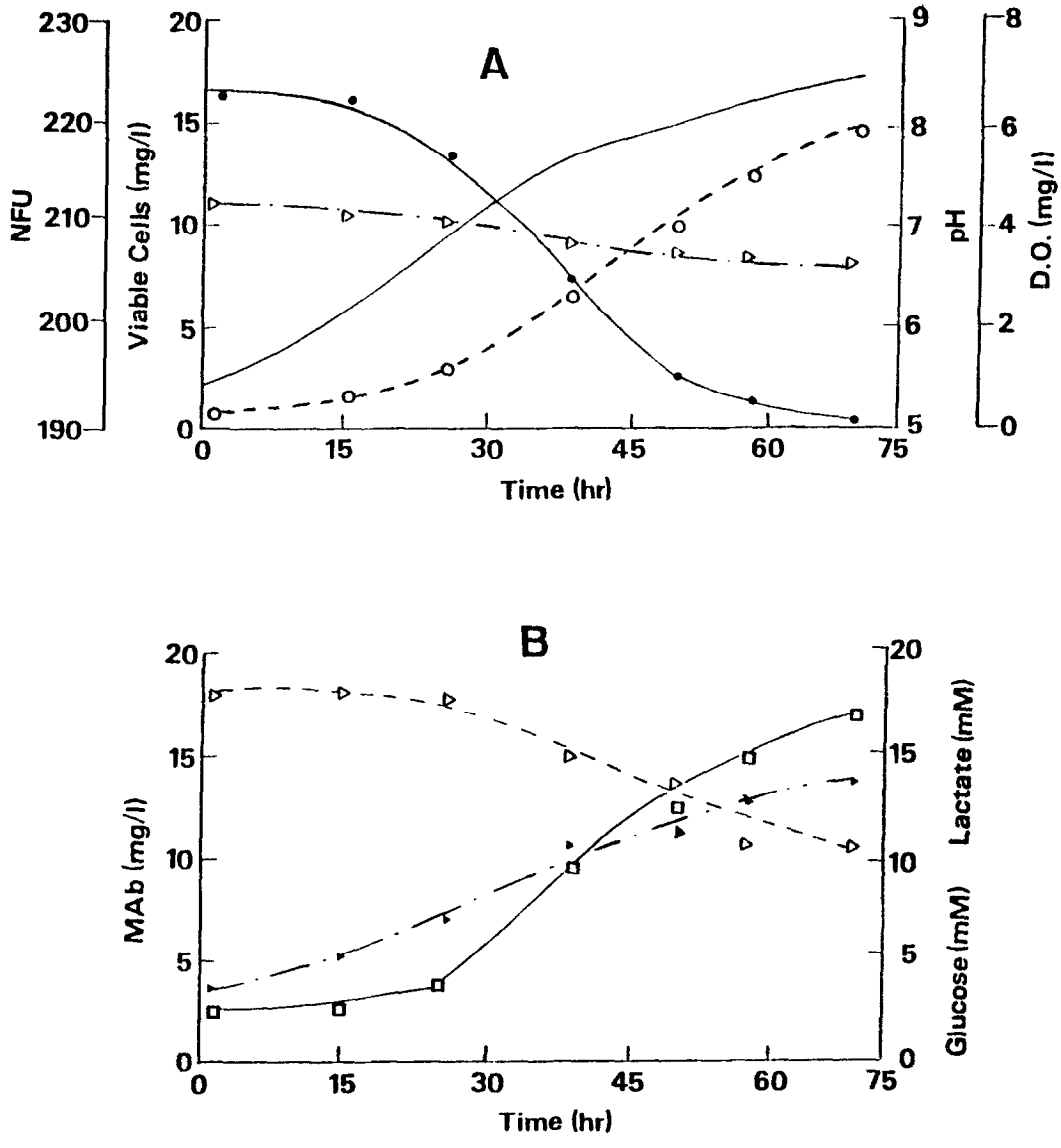


Figure 1. Panel A: Profiles of culture fluorescence [—], pH [ ▷ ], dissolved oxygen [ • ] and viable cell counts[ o ]. Panel B: monoclonal antibodies [ □ ], glucose[ ▷ ], and lactic acid[ ▸ ] for a typical hybridoma culture.

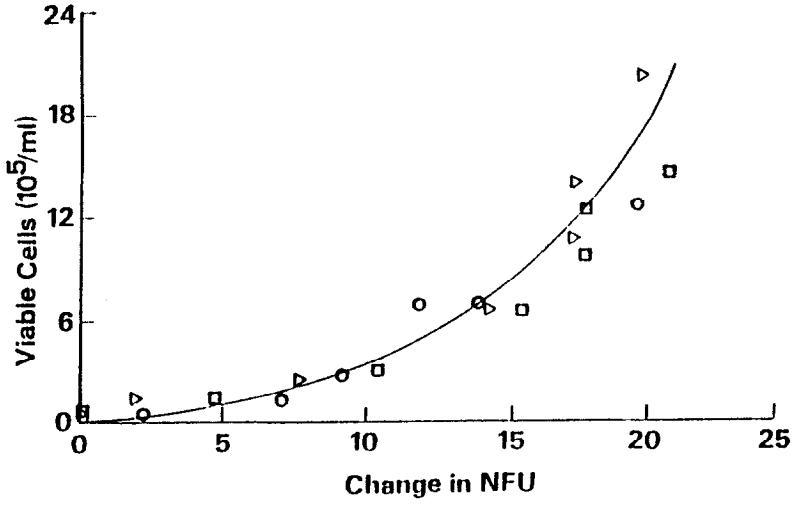


Figure 2. Relationship between cell count and change in culture fluorescence in batchecultures. Batches 1, 2 and 3 are[▫ ], [ o ], and [▷ ] respectively.

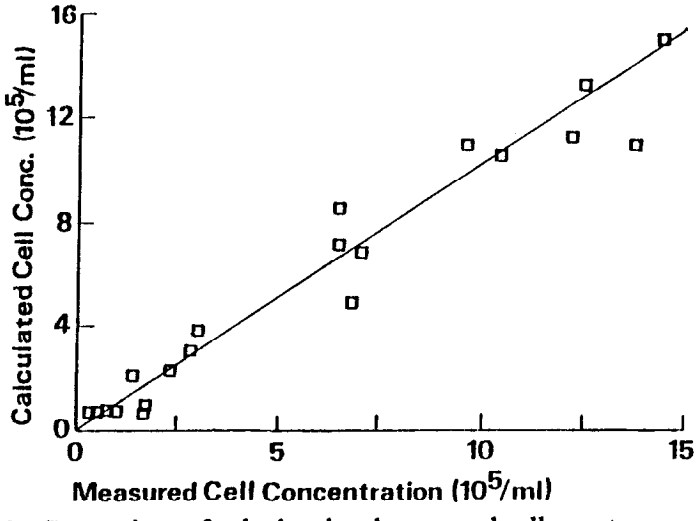


Figure 3. Comparison of calculated and measured cell counts

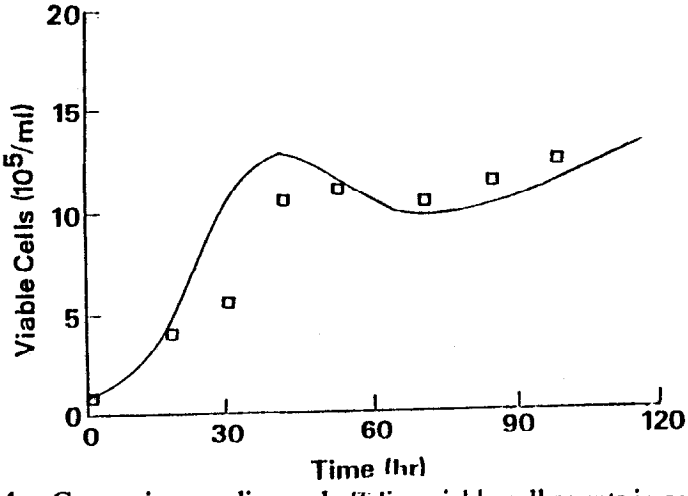


Figure 4. Comparison on-line and off-line viable cell counts in continuous culture. Line refers to calculated values and data points to measured values

concentration increased in proportion to cell count and reached a maximum at about 24 h after the viable cell count attained its maximum.

In Figure 2, a plot of the cell concentration (N) is given as a function of the increase in culture fluorescence (ΔNFU) for three separate batch experiments. The data presented in this figure are during the exponential growth phase of the hybridoma. The variable, ΔNFU, is defined as:

$$\Delta NFU = NFU(t) - NFU(t=0) \qquad (1)$$

where NFU (t=0) is the initial culture fluorescence. The variable ΔNFU quantifies the change in culture fluorescence as a result of increase in cell number in the reactor. Subtraction of the initial culture fluoresence is needed to eliminate batch to batch variations in the fluorescence of the medium. Typical variation was of the order of 5 to 10 NFU.

The data in Figure 2 show a strong correlation of cell number to change in culture fluorescence. The nonlinear response shown in Figure 2 can be fitted to an equation of the form:

$$N = C_1 + C_2 (\Delta NFU) + C_3 (\Delta NFU)^2 \qquad (2)$$

Notice that for a small change in N at low values of N, large changes in fluorescence signal were observed in all experiments conducted. At high viable cell concentrations (greater that $10^6$ cells/ml), the culture fluorescence signal is less sensitive to cell concentration changes. This is due to increased scatter and refraction of incident light (340 nm) caused by increased cell concentration and decreased emitted light reaching the detector. At low N, the fluorescence sensitivity is about 35 NFU/$10^6$ cells and at high values of N, it decreases to 5 NFU/$10^6$ cells. The electronics and optics of the measurement system are designed to provide high signal-to-noise ratio, and therefore, changes in fluorescence output of 1 NFU can be reliably measured. Since hybridomas grow in suspension up to a maximum of about 2 x $10^6$ cells/ml, the culture fluorescence is a viable method for monitoring hybridoma cell concentration in practical systems.

A nonlinear regression to minimize squared error was performed and optimum values of $C_1$, $C_2$, and $C_3$ were found as: $C_1$ = 82,200, $C_2$ = - 8,944, $C_3$ = 3,605. The coefficient of determination and correlation were 0.948 and 0.974, respectively. The cell numbers predicted by Eq. 2 are compared with experimental values in Figure 3. The comparison suggests that the correlation of the form given in Eq. 1 provides for a reasonable framework for relating cell growth to changes in culture fluorescence. The relative error in estimating cell count from fluorescence measurement is 40% at low concentrations

and 80% at high concentrations.

The ability to estimate hybridoma cell concentration on-line from culture fluorescence data provides a convenient means for starting a continuous flow bioreactor after the initial batch growth phase. In order to examine the suitability of this approach, a continuous reactor experiment was conducted. In Figure 4, the reactor was run in a batch mode for the first 48 h, following which the nutrient feed pump was tuned on with concomitant removal of an equivalent volume of the reactor contents so that the hold-up in the reactor remained constant at 2.5 liters. The cell count continued to increase to 1.2 X $10^6$ cells/ml and remained near this value for the next 100 h. The cell counts estimated using the measured culture fluorescence values in Eq. 2 are compared with the experimentally determined values in Figure 4. Reasonable agreement suggests that the approach of using culture fluorescence to monitor cell count is a useful method. Since chemostats are susceptible to washout, a situation which occurs when the nutrient renewal rate exceeds cell growth rate, the on-line means of estimating hybridoma cell count using culture fluorescence provides for a convenient method for regulating the nutrient feed pump so that automatic corrective actions for potential washout situations can be implemented.

## CONCLUSIONS

Culture fluorescence provides a convenient in situ method for measuring hybridoma growth. This approach can be used for on-line monitoring of industrial bioreactors employing hybridomas.

## ACKNOWLEDGEMENTS

This project is supported by the Commonwealth of Pennsylvania's Ben Franklin Partnership through the Advanced Technology Center of Southeastern Pennsylvania. The ATC is a program of the University City Science Center in Philadelphia, PA, USA.

## REFERENCES

Armiger, W. B., Forro, J.R., Maenner, G. F. & Zabriskie, D. W. (1984). Proceedings of Biotech '84, Washington, D.C., 601-628.

Armiger, W.B., Forro, J.R., Montalvo, L.M., Lee, J.F. & Zabriskie, D.W. (1986). Chem. Eng. Commun., **45**, 197-206.

Beyeler, W., Einsele, A. & Fiechter, A. (1981). European J. Appl. Microbiol., **13**, 10-14.

Bosworth, J., Brimfield, A., Naylor, J. & Hunter, K. (1983). J. Immun. Meth., **62**, 331-336.

Duysens, L.N.M. & Amesz, J. (1957). Biochim. Biophys. Acta., **24**, pp. 19-26.

Harrison, D.E.F. & Chance, B. (1970). Appl. Microbiol., **19**, 446-450.

Henry, R. J. (1968). Clinical Chemistry: Principles and Techniques, Harper & Row, NY., 664-666.

Hyvarinen, A. & Nikkila, E. (1962). Clin. Chem. Acta., **7**, pp. 140.

Luong, J.H.T. & Carrier, D.J. (1986). Appl. Microbiol. Biotechnol., **24**, pp. 65-70.

MacBride, W.R., Magee, J.A., Armiger, W.B. & Zabriskie, D.W. (1986). U.S. Pat. 4,577,110.

Ozato, N., Mayer, N. & Sachs, D. (1980). J. Immunol., **124**, 533-540.

Ristroph, D.L., Watteeuw, C.M., Armiger, W.B. & Humphrey, A.E. (1977). J. Ferment. Technol., **55**, 599-608.

Zabriskie, D.W., & Humphrey, A.E. (1978). App. Environ. Microbiol., **35**, 337-343.