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Pericellular oxygen depletion during ordinary tissue culturing, measured with oxygen microsensors

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Abstract. Recent research has found important differences in oxygen tension in proximity to certain mammalian cells when grown in culture. Oxygen has a low diffusion rate through cell culture media, thus, as a result of normal respiration, a decrease in oxygen tension develops close to the cells. Therefore, for the purpose of standardization and optimization, it is important to monitor pericellular oxygen tension and cell oxygen consumption. Here, we describe an integrated oxygen microsensor and recording system that allows measurement of oxygen concentration profiles in vertical transects through a 1.6-mm deep, stagnant, medium layer covering a cell culture. The measurement set-up reveals that, when confluent, a conventional culture of adherent cells, although exposed to the constant oxygen tension of ambient air, may experience pericellular oxygen tensions below the level required to sustain full oxidative metabolism. Depletions reported are even more prominent and potentially aggravating when the cell culture is incubated at reduced oxygen tensions (down to around 4% oxygen). Our results demonstrate that, if the pericellular oxygen tension is not measured, it is impossible to relate *in vitro* culture results (for example, gene expression to the oxygen tension experienced by the cell), as this concentration may deviate very substantially from the oxygen concentration recorded in the gas phase.

INTRODUCTION

It has long been acknowledged that the lower limit of oxygen partial pressure for full cellular respiration of Erlich ascites cells in culture is 0.13 kPa (1300 p.p.m. or 1.3% O_2) (Froese 1962). This limit is determined simply by limitation in the access to oxygen molecules that cells have at lower oxygen concentrations, because of the low diffusion rate of oxygen in aqueous solutions (Boag 1970).

Recent developments have shown that cells in culture and in tissues may also respond to more moderate hypoxia by a complex gene-regulatory responses involving selective activation of many genes, most of which are influenced by the hypoxia-responsive factor (HIF) (for surveys please see Ebbesen *et al*. 2004 or Hopfl *et al*. 2004).

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In cell culture experiments, control of pericellular oxygen tension is traditionally achieved by fixing the oxygen tension in the gas phase above the medium (Wolff *et al*. 1993; Mamchaoui & Saumon 2000). However, pericellular tension also depends on whether or not the medium is stirred, the number of cells and their metabolic rate in a given culture as well as the diffusion distance from the medium surface to the flask bottom (Chapman *et al*. 1970).

Here we report on: (i) our equipment and methods making it possible to constantly monitor pericellular oxygen tensions and even detect local effects of individual cell colonies, (ii) a drastic decrease in pericellular oxygen tension during the normal course of a single passage of a cell culture, despite controlled high and constant oxygen concentration in the gas phase, (iii) the oxygen consumption rate per cell as calculated from oxygen tension profiles in the medium.

MATERIALS AND METHODS

Cells

Cells of the human breast cancer cell line T-47D (Keydar *et al*. 1979) were grown as monolayer cultures in RPMI 1640 medium (Gibco, Rockville, MD, USA), supplemented with 10% foetal calf serum (Gibco), 2 mM L-glutamine (Gibco), 200 units/l insulin and 1% penicillin/streptomycin (Gibco). The median doubling time for T-47D cells under conditions of ambient air was 37 h (Stokke *et al*. 1993). T-47D cells express wild-type pRB, but the p53 gene is mutated in codon 194 (Casey *et al*. 1991). Cells (10_6) were seeded in 5 ml medium on day 0 in 25-cm^2 Primaria flasks from Beckton Dickinson (Franklin Lakes, NJ, USA), and were incubated in an In Vivo2400 glove box hypoxia workstation at 4 or 19% oxygen with 5% CO_2. Culture flasks had their caps open for gas exchange, but were equipped with filters to prevent entry of micro-organisms.

Experimental protocol

The technology platform for measuring oxygen tension in the medium above adherent cultured cells was set up with visual display of the measuring electrode tip position. Thus, all measurements took place without removing cultures from the incubator. Seeding density of the cells was 5×10^5cells in 25^2 flasks, gas phase was 4 or 19% and monitoring was undertaken for 5 or 7 days, measuring each culture daily, once with the electrode aimed vertically down, and once in the opposite direction.

Measurement set-ups

The set-up for measuring microgradients of oxygen (for an overview, see Fig. 1) was purchased from Unisense A/S, Aarhus, Denmark. It consisted of an oxygen microsensor, held by a motorized and computer-controlled micromanipulator, mounted on an inverted microscope inside the incubator. The oxygen microsensor (OX10 Clark-type) had a tip diameter of 10 μM (Fig. 2) and a guard-cathode (not shown on figure) to prevent diffusion of oxygen from the electrolyte reservoir reaching the oxygen-sensing cathode (Revsbech 1989). The sensor was filled with electrolyte to shield signals from interfering with electrochemical fields, thus obviating the use of a noise-shielding Faraday cage. In use, oxygen diffuses through a silicone tip membrane to an oxygen-reducing cathode, which is polarized against an internal Ag/AgCl anode. Flow of electrons from the anode to the oxygen-reducing cathode linearly reflects oxygen partial pressure immediately proximal to the sensor tip. The weak electrical current generated (10^{-12}–10^{-10} amps) is measured by a sensitive picoammeter PA2000. The picoammeter comes with two channels, and has a built-in polarization source.

Figure 1. The set-up for measuring oxygen tension is shown in place inside the incubator. The oxygen microsensor is held by a motorized micromanipulator mounted on a metal frame on the inverted Olympus CK41 microscope.

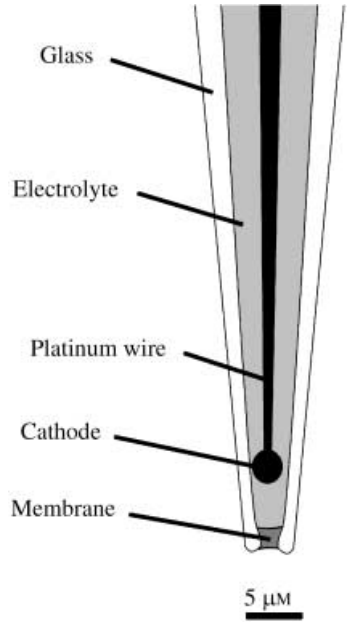

Figure 2. The sensor tip.

The oxygen microsensor was positioned with the motorized micromanipulator (MMS) mounted on a metal frame on the inverted Olympus CK41 microscope (Fig. 1). The motorized micromanipulator was controlled with a motor controller (MC-232) connected to a laptop computer running the Unisense Profix 3.05 data acquisition software.

The culture to be studied was placed on a manually operated XY microscope stage on the inverted microscope, inside the incubator. Flasks were placed permanently under electrodes, there was no stirring, and all measurements were performed inside the incubator. Readings were monitored on screens outside the incubation box, the microscope being equipped with a digital video camera. Pictures were acquired and stored directly by an Olympus DP-Software program. As measurements were performed with the bottle caps removed, the sensor could be inserted with an inclination of 30° without disturbing the media.

Sensor calibration

For the daily experiments, an initial 2-point calibration was performed. The zero reading was obtained in a saline alkaline/sodium ascorbic acid solution with both ascorbic acid and sodium hydroxide at final concentrations of 0.1 M. As oxygen partial pressure was controlled in the incubator box, a second calibration point was obtained in a solution in equilibrium with the atmosphere in the box. Calibrations were obtained by use of the calibration module of the Profix data acquisition software.

Measurement of oxygen consumption

Steady state linear oxygen gradients were established in the culture flasks with a slope that was proportional to the respiration rate of the cells in the culture flasks. Oxygen concentration gradient generated by cells attached to the bottom of the culture flasks, was determined by measuring the oxygen concentration at consecutive equidistant measurement points down through the 1.6-mm thick layer of aqueous medium covering the cells at the bottom of the flasks. Oxygen concentration gradients were resolved in steps of 25 μm. Measurements were performed both descending through the medium to the cells at the bottom and re-ascending through the medium. No hysteresis effect was observed. The software allowed automatic profiling with fixed time intervals for several days. Location of the sensor was followed and photographed via the microscope.

Sensor tips were disinfected inside the glove box after each reading by rinsing first in sterile water followed by 5 minutes' submersion in 70% ethyl alcohol.

Calculation of respiration rate based on oxygen concentration profiles

The thin, 1.6-mm, layer of medium, the lack of air circulation above and the temperature homogeneity of the system gave rise to a stagnant medium above the cells. Oxygen transport through this stagnant layer was thus entirely based on molecular diffusion, as there was no turbulence, laminar flow or other bulk movements of the media. Oxygen consumption by the cells at the bottom of the flask resulted in a linear concentration gradient through the medium. The microsensor had insignificant oxygen consumption and thus did not affect oxygen gradients.

By using Fick's 1st law of diffusion (Crank 1975), the oxygen flux towards the cell layer can be estimated from the linear oxygen gradient measured by the sensor.

$$J = -D\frac{dC}{dX} \quad (1),$$

where dC/dX is the slope of the gradient, and D is the diffusion coefficient of the medium. Under steady-state conditions, the oxygen flux will be equal to the respiration rate of the cells covering the flask bottom.

Measurements between a depth of 400 μm below the medium surface and 100 μm above the flask bottom, which displayed an essentially linear depth-O_2-concentration relationship, were used to determine the oxygen concentration gradient by linear regression. The oxygen consumption rates (nmol/h/cm^2) were calculated, assuming that the solubility of oxygen in the medium (approximately 9‰ salinity and 37.0 °C) was 200 μmol/l (Garcia & Gordon 1992). The diffusion coefficient in culture media at 37.0 °C is approximately 3.37×10^{-5} cm^2/s. An Excel spreadsheet to automate these calculations can be downloaded from Unisense's website at http://www.unisense.com.

Respiration rates were expressed in fmol O_2/h/cell by dividing the obtained rates of respiration per cm^2 (according to Equation 1 above) by the number of cells per cm^2. The cells of each bottle were detached by means of trypsin treatment before counting in a Neubauer chamber.

RESULTS

The linearity of the reading down to very low oxygen tension is demonstrated in Fig. 3. Figure 4 is a photograph of the sensor tip precisely touching cells at the bottom of the flask. Oxygen profiles (that is concentration of oxygen as a function of height above the flask bottom) on days 1, 2, 3 and 4 after seeding of 0.5 million cells on a 25-cm^2 flask bottom, and on days 4 and 7 after seeding of 1 million cells are shown in Fig. 5. Cultures seeded with 0.5 million cells on day 0 were subconfluent by day 4 and showed an even distribution over the flask bottom with small open areas between colony-like assemblies of cells (Fig. 6). Cultures seeded with 1 million cells were certainly confluent by day 5 and verging to super-confluence by day 7 (Fig. 7); being a tumour cell line, it was not surprising that the T-47D cells were not contact inhibited. In Fig. 5, shifts can be seen in the uppermost areas of the profile where the sensor tip moves from gas phase to its first contact with the medium surface. In the lower areas of the profiles, for days 3 and 4, linear oxygen profiles can be seen where the measuring tip approached the bottom of the flask between cell colonies (that is, in an open area), but were curved when measurements were taken from the surface of the cell colonies. These results demonstrate the range of measurement of sensitivity achievable with our sensor.

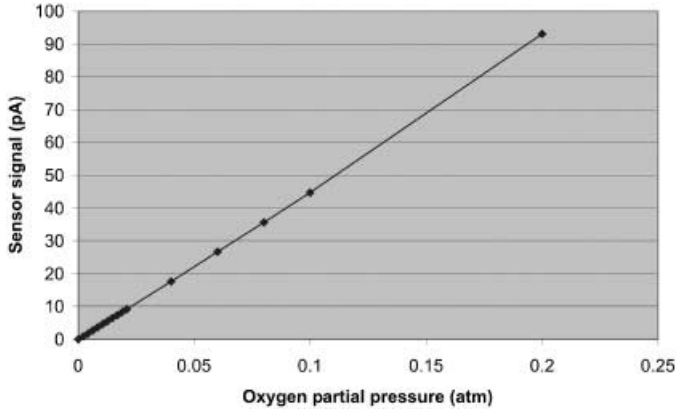

Figure 3. Demostrates the liniar response in picoampere of an oxygen microsensor as a function of the oxygen partial pressure around the sensor tip.

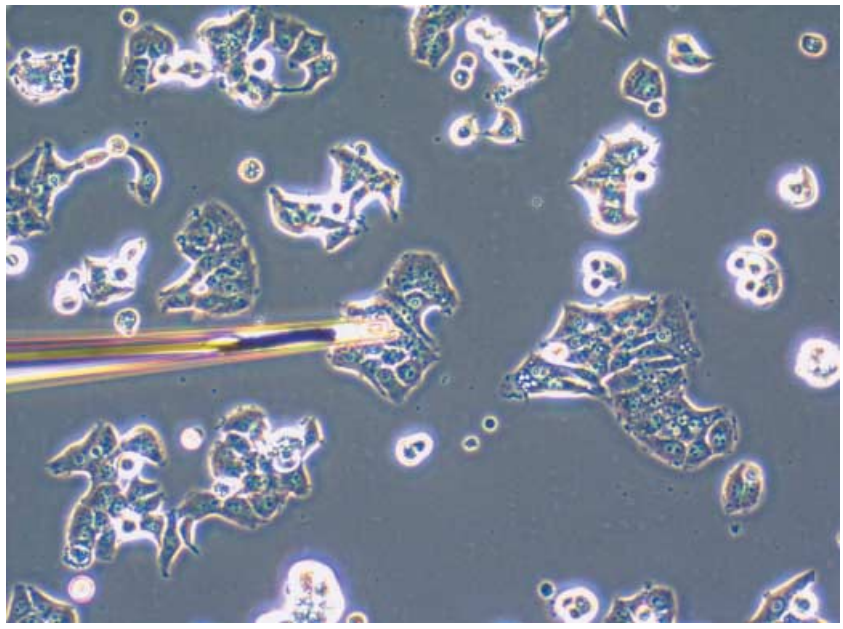

Figure 4. The sensor tip seen in contact with a small colony of cells.

Cultures in 19% oxygen in the ambient air gas phase showed that within 7 days of culture, pericellular oxygen tension may change from *in vivo* physiological hypoxia values, to non-physiological levels of hypoxia.

Oxygen profiling with 19 and 4% oxygen in the gas phase indicated similar (parallel) decreases in pericellular tension, and also similar increases in the difference between surface and base tensions during five days of culture (Fig. 8 and Table 1a). Figure 8, however, shows that surface-base differences increased somewhat more after 4 days of culture in 19% oxygen than in 4% oxygen gas phase/oxygen tension. This may well indicate that access to oxygen was insufficient for maximum respiration with 4% O_2, while it was still sufficient with 19% O_2.

Calculation of oxygen consumption showed the expected increase, with increasing cell number during culture. In addition, we found declining oxygen consumption per cell when cultures became very dense (Table 1b).

Use of the microsensor under these careful conditions, resulted in no incidences of culture infection.

DISCUSSION

The horizontal shift in the first (uppermost) parts of the gas tension profiles, demonstrated in Fig. 5, reflect the transition point at which the sensor tip makes its first contact with the surface of the medium.

Two different phenomena explain the sudden decrease in oxygen tension:

1 The moment the sensor touches the medium surface, a fluid layer is attracted around the sensor tip by surface tension; this creates a local surface 'bump'. The first measurements within the medium are therefore recorded relative to a depth of a few μm below the surface,

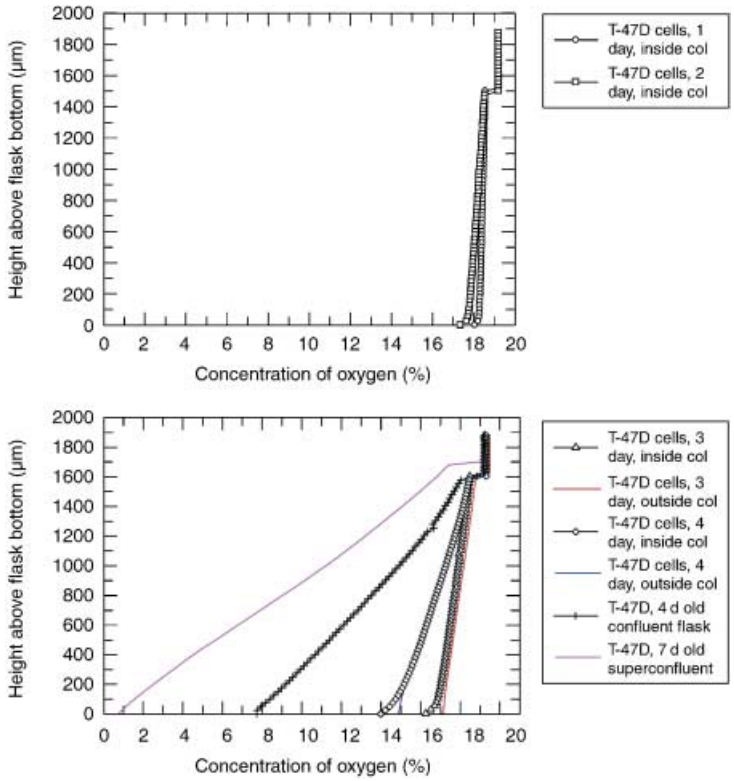

Figure 5. Oxygen tension profiles (i.e. oxygen tension as a function of the height above the flask bottom) obtained with conventional cultures of T-47D cells. At day 0 5×10^6 or 10^2 cells were seeded per flask and threafter grown in an atmosphere of ambient air with 5% CO_2. Symbols ○ and □ (a) and △ and ◇ (b) represent, respectively, 1-, 2-, 3- and 4-day-old cultures with 0.5×10^6 cells seeded per flask at day 0. All these profiles represent measurements where the measuring tip descended directly on top of a colony. However, for the 3- and 4-day-old cultures, measurements are shown where the measuring tip ended between colonies. These measurements are shown as fully drawn lines and appear visible at the base of the symbol curves. Symbol + and the fully drawn line without symbols, shown in (b) represent, respectively, 4- and 7-day-old cultures with 10^6 cells seeded per flask. Notice the low pericellular oxygen tension at the end of the culture period for the confluent cultures on days 4 and 7.

representing concentration at a depth of some µm. When the sensor tip is applied deeper into the fluid, the empirically derived 30° angle of afferent inclination causes this surface tension effect to disappear. Thus, oxygen gradients in equidistant steps in the upper 400 µm of fluid in the column cannot be accepted as true measurements and, for this reason, these parts of the curves were not used for final calculations. The artefact described is visible on curves (Fig. 5), where gradients in the upper 400 µm are less steep than those measured at lower depths.

2 The oxygen microsensor gave 4% higher signals in the gas phase, compared with stagnant fluid at equal oxygen partial pressure. Oxygen partial pressure measured in the gas phase was therefore overestimated by 4%, relative to the calibration for in stagnant fluid. This effect is as a result of the higher diffusivity of oxygen if air is surrounding the sensor tip rather than if the same sensor tip is submerged.

Curving profiles observed when the sensor tip closely approached cell aggregates reflected the true local pericellular gradient in a stagnant layer of culture medium. Curves describing local variations in oxygen tension when the sensor tip was directly above a cell colony, and corresponding

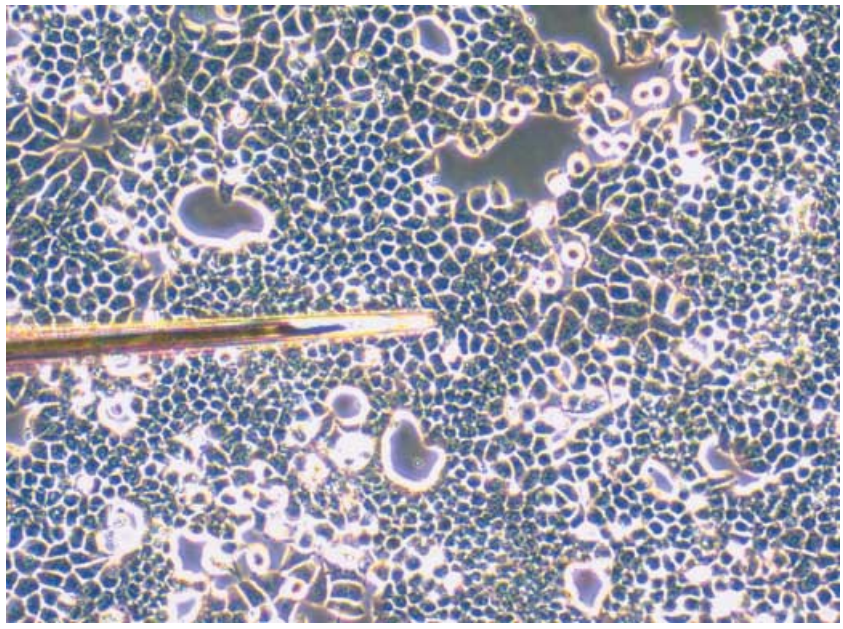

Figure 6. The confluent day 4 culture of T-47D cells. Seeded 10^6 cells per flask and grown at 19% O_2.

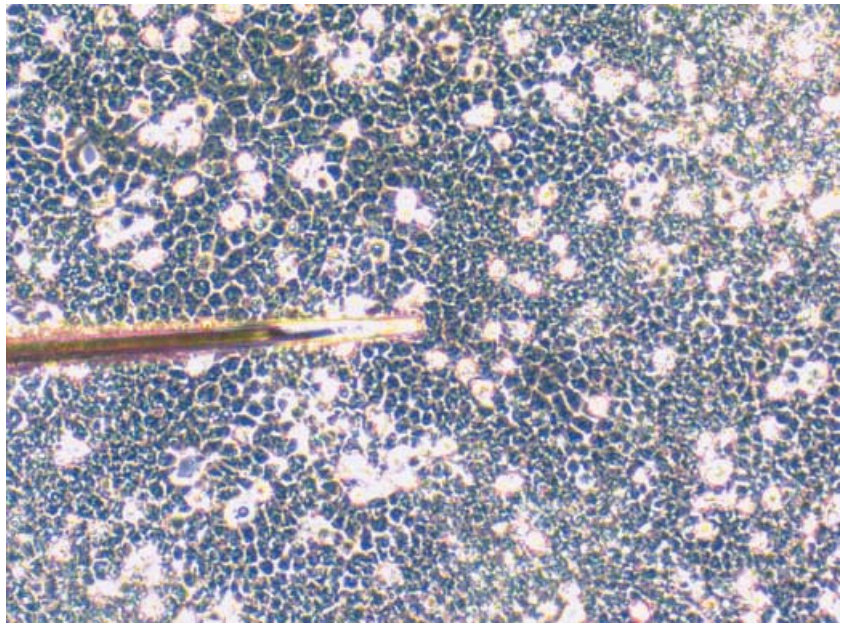

Figure 7. The superconfluent day 7 culture of T-47D cells. Seeded 10^6 cells per flask and grown at 19% O_2.

to proximity to cell aggregates, were far more prominent than at higher levels the medium. Higher above the base profiles became linear; the gradient reflecting average cell density in a larger area rather than local concentration gradients as a result of a heterogeneous distribution of cells.

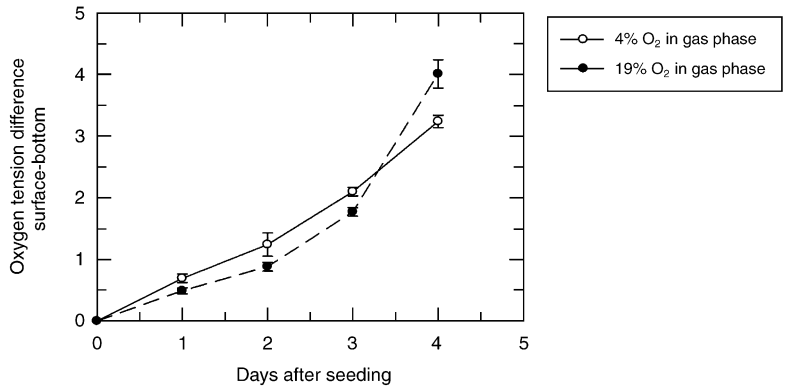

Figure 8. Oxygen tension differences surface-bottom shown as a function of days after seeding 5×10^5 cells/flask. The day 7 culture reaches a pericellular tension below the level of oxydative respipation (1.3%). The open circles show cells growing in an atmosphere with 4% oxygen and closed circles in an atmosphere with 19% oxygen.

Table 1a. Culture going from subconfluency to confluency. Comparing the development in the pericellular oxygen concentration and the differences in oxygen concentration between the gas phase and at the cell membrane for T-47D cells grown at 20 and 4% oxygen for 5 days and oxygen consumption of the culture cells on day 4. On day 0, 5×10^5 T-47D cells were seeded in 25-cm^2 Primaria flasks. One flask was used for consecutive oxygen profiles, while one was used as control with only one profile on day 4. Number of cells was determined on day 4. Here, the first measurements after the sensor had reached the media surface, and the last measurements when the sensor had reached the bottom of the flask are shown, together with the difference between the two. All profiles ended up outside of colonies, and all values except cell number are in per cent oxygen

Gas phase	Test no.		Day 1	Day 2	Day 3	Day 4	Day 4 control	Cells day 4 $\times 10^6$	Consumption fmol/h/cell
4% O_2	1	Top	3.87	3.90	3.68	3.52	3.51	3.00	
		Bottom	3.24	2.86	1.69	0.54	0.53	Contr.: 3.00	227
		Difference	0.63	1.04	1.99	2.98	2.98		
4% O_2	2	Top	4.00	4.01	3.96	4.18	3.85	4.28	
		Bottom	3.40	2.96	1.75	0.60	0.40	Contr.: 3.95	174
		Difference	0.60	1.05	2.21	3.58	3.45		
4% O_2	3	Top	3.73	3.87	3.77	3.50	3.51	5.09	
		Bottom	2.90	2.25	0.74	0.31	0.26	Contr.: 5.30	130
		Difference	0.83	1.62	2.03	3.19	3.25		
19% O_2	4	Top	19.12	17.97	18.76	18.38	18.22	3.38	
		Bottom	18.68	17.16	17.06	14.84	14.46	Contr.: 3.35	208
		Difference	0.44	0.81	1.70	3.54	3.74		
19% O_2	5	Top	18.91	19.19	19.15	18.92	18.80	4.52	
		Bottom	18.37	18.24	17.32	14.71	14.24	Contr.: 4.89	177
		Difference	0.54	0.95	1.83	4.21	4.56		

The difference in oxygen tension between the medium surface and the flask bottom was little affected by the gas phase tension, as demonstrated in Fig. 8. This was to be expected, as the pericellular oxygen tension in these cultures always remained above the level for full respiration (approximately 0.1% O_2). However, Fig. 8 indicates that the difference was higher and, thus, that

Table 1b. High cell density cultures. Confluence was reached on day 4 and superconfluence on day 7 since originally seeded with 1 million cells in 25-cm^2 flasks and 19% O_2 in gas phase

Confluent cultures		Oxygen level (%)	No. of cells $\times 10^7$	Oxygen consumption (fmol/h/cell)
Confluent culture	Top	17.63	1.37	146
	Bottom	7.51		
	Difference	10.12		
Super-confluent culture	Top	17.39	2.49	127
	Bottom	0.75		
	Difference	16.64		

the gradient became a little more pronounced after 4 days of culture in 19% oxygen as compared with with 4% oxygen in their atmosphere. This may well be related to the local gradient around cell colonies being more pronounced than the overall gradient higher up in the flask. If local gradients vary greatly, this may indicate a lack of access to oxygen after 4 days under 4% O_2 in the atmosphere, while there was no such lack after 4 days under 19%.

Our oxygen microsensor system with programmed decent of the tip provides profiles permitting calculation of both the pericellular oxygen tension and oxygen consumption. As the readings can be programmed to be repeated at fixed time intervals, pericellular oxygen tension and oxygen consumption measurements may become a standard parameter of tissue culturing, where a stable cell environment is important for reproducibility of experiments and/or for optimizing the output of a cellular product. Our system of monitoring classical cell cultures, growing attached to a flask bottom, clearly demonstrates that if the variation in pericellular oxygen tension is not determined, one cannot relate *in vitro* culture results to any defined oxygen tension by simply giving the constant gas phase value used during the culturing.

Of note, the mean oxygen consumption per cell per hour on day 4 of the study was only slightly lower for the subconfluent cultures where the gas phase contained 4% oxygen as compared with 19% oxygen, but super-confluent cultures, grown with 4% oxygen in the gas phase, showed reduced respiration per cell, although the pericellular oxygen concentration was in this case somewhat higher (0.31% oxygen) than the lower limit for full respiration previously reported by Froese (1962). Testing confluent human foreskin cultures, we found a much lower oxygen consumption than for T-47D cells, and for NHIK 3025 cells (results not shown). Pericellular oxygen tension has been measured in a number of previous experiments, and its importance has been realized (Metzen *et al*. 1995). In this study, our contribution is a quantification of oxygen tension profiles through undisturbed culture media, and their change with increase in size of adherent cell populations. Such recordings could, in principle, be used for changing gas phase oxygen tension in order to counter any decline in pericellular oxygen tension during culture growth, with the purpose of keeping a constant oxygen environment (work in progress).

CONCLUSION

An integrated oxygen microsensor and recording system for measuring both pericellular oxygen tension and the oxygen flux, in vertical transects of tissue culture medium, has been described. It has allowed determination that during growth of adherent cells to confluency, pericellular

oxygen tension may descend below the level required for full cellular oxygenation, in spite of constant and sufficient oxygen tension in the gas phase. Considering the last decade's finding of a close relationship between oxygen tension and the intimate cellular environment, it means that standardization and comparison of results from different cultures requires direct measurements of pericellular oxygen tension.

ACKNOWLEDGEMENTS

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