REGULATION OF TISSUE pH IN PLANTS AND ANIMALS
A REAPPRAISAL OF CURRENT TECHNIQUES

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CHRISTOF J. SCHWIENING

Measurement of intracellular pH: a comparison between ion-sensitive microelectrodes and fluorescent dyes

Introduction

Intracellular pH (pH_i) has been studied extensively for over a century using a wide range of techniques. These techniques have been subject to constant improvements to the extent that useful measurements can now be made in even the smallest of cells. This chapter outlines the development of ion-sensitive microelectrodes and dyes and highlights some of the difficulties and dangers of both of these methods. It then concentrates on some recent advances in our understanding of how fluorescent pH-sensitive dyes might best be used. Finally, a tabulated comparison between the two techniques is presented.

Historical perspective on pH_i measurement

Although pH_i changes had been observed much earlier, the first measurements of what could loosely be described as intracellular pH were made around 1910 using cell extracts and platinum/hydrogen electrodes (for a review see Caldwell, 1956). For example, in 1912 Michaelis and Davidoff measured the pH of blood and noted that red cell lysis caused a change in bulk pH. The technique of cell lysis was perhaps most suited to the measurement of pH_i in non-nucleated erythrocytes where intracellular compartments did not complicate the measurements. At around the same time various workers were using naturally occurring pH indicators to visualise changes in pH_i (e.g. Crozier, 1918). The problems associated with such indirect techniques were recognised very early on and the search for better methods led in three directions: distribution of weak acids and bases; smaller electrodes to allow direct measurement of pH_i; and better indicators and techniques to load them into cells.

Weak acid and base distribution

Detailed discussion of the distribution of weak acids or bases is beyond the scope of this chapter but readers who are interested should consider

the review by Roos and Boron (1981). In broad terms, the distribution of the weak acid or base across the plasma membrane is dependent upon the pH in both the extracellular and intracellular compartments. Fridericia (1920) was the first to use this technique to measure the pH_i of red blood cells using CO_2 and he obtained a pH_i of 7.30. Later dimethyloxazolidine-dione (DMO; Waddell & Butler, 1959) was introduced as a pH_i indicator. Although the technique is easy, accurate and can be applied to small cells, its use was limited by the fact that it requires the destruction of the tissue and therefore cannot easily be used to measure pH_i changes over time. The technique also has a major problem in that it can alter pH_i. However, the principle of weak acid or base permeation and equilibration across the cell membrane is of some interest. De Vries (1871) first showed colour changes in living beetroot when exposed to ammonia. The colour changes reflected changes in intracellular pH as the uncharged, lipid-soluble weak base diffused across the cell membrane and then associated with H^+ causing a profound alkalinisation. The movement of uncharged weak acids or bases therefore not only allowed pH_i to be estimated, but was also a powerful means of adding or removing acid equivalents from cells. The application of weak acids or bases to cells has become the ubiquitous method for challenging and thereby studying pH_i regulation.

pH-sensitive electrodes

Early electrodes

Intracellular pH-sensitive electrodes have been made out of numerous materials. They can be broadly divided into three groups:

metals: platinum–hydrogen electrodes (Taylor & Whitaker, 1927); antimony (Buytendijk & Woerdeman, 1927); tungsten (Caldwell, 1954);
glass: (Caldwell, 1954; Hinke, 1967; Thomas, 1974);
liquid membranes: bicarbonate sensor; nigericin (Matsumura *et al*., 1980); neutral H^+ exchanger tri-n-dodecylamine (Ammann *et al*., 1981).

High electrical resistance, however, presented a major problem for the miniaturisation of metal minielectrodes. It was not until the 1950s that significant progress was made in producing microelectrodes that could be widely used. pH-sensitive glass, although discovered around 1900, became the material of choice. It has relatively low electrical resistance, no sensitivity to oxidising and reducing agents, dissolved

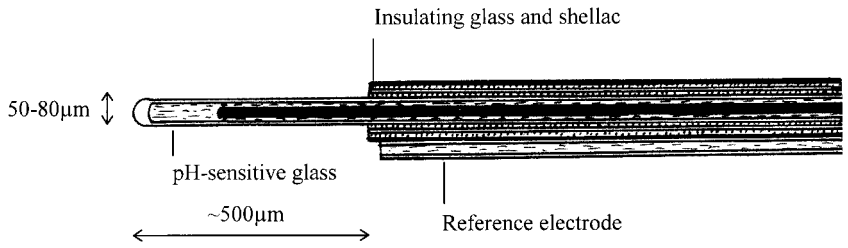

Fig. 1. Glass pH-sensitive electrode used by Caldwell (1954) to measure pH_i in crab muscle fibres. The unexposed pH-sensitive (low-resistance) glass was insulated with shellac and insulating glass. In many cases, Caldwell used wax to attach a reference electrode. Modified from Caldwell (1954).

gasses, anions or buffers, and it is stable and can produce a relatively rapid response.

Only two particular microelectrode types are concentrated on in this section – the combination electrode, pH and reference, produced by Caldwell in 1954 (Fig. 1) and the recessed-tip design produced by Thomas in 1974 (Fig. 2). The Caldwell electrode was a design classic but is no longer in use because its relatively large size restricted its use to large cells such as crab muscle fibres. The electrode consisted of a portion of exposed pH-sensitive glass about 500 μm long. Such a long length of what was termed 'low-resistance glass' was necessary to obtain usable resistance of 1 GΩ. The unexposed length was insulated with pH-insensitive glass and shellac. This design was modified by

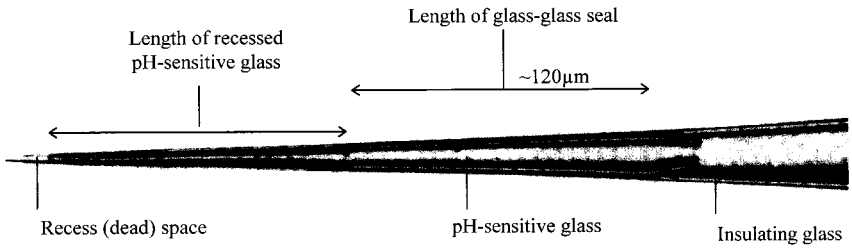

Fig. 2. Recessed-tip pH-sensitive microelectrode as used by Thomas (1974) to measure pH_i in snail neurons. A microforge was necessary for the construction of these electrodes. A heated filament was used to soften the low-melting-point, inner, pH-sensitive glass. Simultaneous application of high pressure down the pH-sensitive glass caused a high-resistance glass-to-glass seal to form.

Hinke (1967), who elegantly replaced the shellac with a glass-to-glass seal, but in the process had to abandon the integral reference electrode. Improvements in both the pH-sensitive glass and the input impedance of modern electrometers allowed the exposed length of pH-sensitive glass to be reduced to about 100 μm, but this still restricted the use of these electrodes to giant cells. However, in cells such as the squid giant axon and barnacle muscle fibres these electrodes are still the method of choice for measuring pH_i.

The first true pH-sensitive *micro*electrode was produced in 1974 by Thomas. Thomas introduced a design whereby the exposed length of pH-sensitive glass (still at least 100 μm in length) was recessed within the insulating glass such that the pH of the recessed space was measured. The result was that only the 1–2 μm tip had to be placed within the cell. Such microelectrodes require considerable skill to manufacture. The recessed-tip pH-sensitive microelectrode has been used to measure pH_i in numerous cell types (snail neurons, skeletal muscle and cardiac muscle) and, despite its relatively slow response, for those who can produce them, it remains the method of choice for measuring pH_i in cells around 100 μm in diameter. Even with such large cells, the requirement to place two microelectrodes into one cell can be difficult to fulfil, especially where the cell boundaries are obscured. Although double-barrelled recess-tip electrodes partly overcame this problem, their construction was too difficult for all but a few (de Hemptinne, 1979).

The problems of slow response, large tips and difficult construction of recessed-tip pH-sensitive microelectrodes were partially solved by the development of liquid ion-exchange microelectrodes.

Liquid ion-exchange pH-sensitive electrodes

The first liquid ion-exchange resin used to measure pH_i was a bicarbonate-sensitive exchanger (Khuri, Bogharian & Agulian, 1974). The microelectrodes had tip sizes of less than 1 μm and had a rapid response time. However, they were also sensitive to carbon dioxide. Nonetheless, they were used to measure pH_i in a number of cell types. Further progress was made in the early 1980s, resulting in neutral carrier microelectrodes based on tri-n-dodecylamine (see Ammann, 1986). There is now a wide range of hydrogen ionophores available with varying characteristics; however, they all share some basic properties.

Using pH-sensitive microelectrodes

The voltage measured from a pH-sensitive microelectrode when placed inside a cell is the sum of both the membrane potential and a potential

sensitive to pH. In order to measure pH_i it is therefore also necessary to measure the membrane potential. This requires either two separate electrodes (Fig. 3A & B) or one double-barrelled electrode (Fig. 3C). The measurement of membrane potential is a major strength of the technique because it provides good information about cell health and electrical activity. Double-barrelled electrodes are more difficult to construct and are generally only used on cells that are difficult to impale with two electrodes. Fig. 3D shows a range of different glass forms that have been used to construct double-barrelled microelectrodes.

Before the organic ligand can be introduced into the micropipette, the glass surface must be made hydrophobic (Fig. 4). This is achieved by baking the micropipette with a silane. There is a wide range of different silanes and protocols used, all with variable success (see Deyhimi & Coles, 1982, for more details). Silanisation both aids the

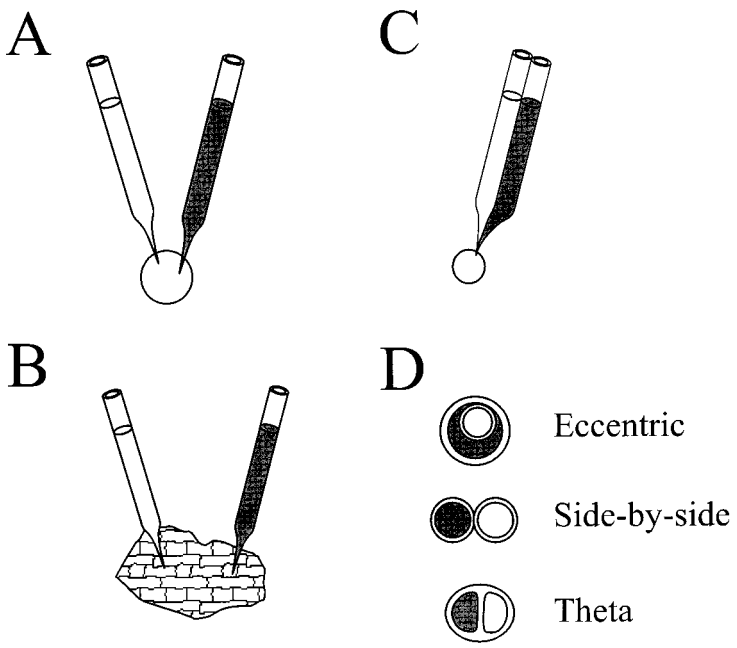

Fig. 3. Microelectrode measurement of pH_i requires the measurement of the membrane potential. This can be achieved in three ways. **A**. Separate electrodes to measure membrane potential and pH placed in the same cell. **B**. Membrane and pH electrodes inserted in different but electrically coupled cells. **C**. A combination pH and membrane potential electrode placed in a cell. **D**. Profiles of the three major double-barrelled electrode configurations.

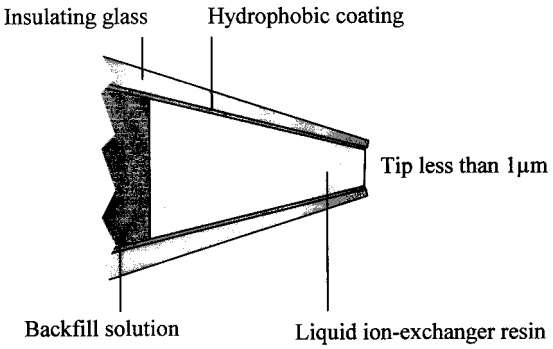

Fig. 4. Liquid ion-exchanger microelectrodes consist of a glass micropipette which is made hydrophobic by treatment with a silane and then filled with a small amount of exchanger resin.

retention of the ligand within the microelectrode tip and reduces the electrical shunt along the glass surface which would otherwise short-circuit the high-resistance ligand. This process is relatively straightforward for single-barrelled electrodes where all of the glass surface can be treated. However, combination electrodes require the selective silanisation of only one barrel of the electrode (see Fig. 3C). Once the electrode is filled with a short column of ligand and a back-filling solution, the electrode has a limited life (usually hours rather than days). Loss of the ligand, blockage of the tip and electrical shunts all have the potential to cause the electrode signal to drift and respond in a sub-Nernstian fashion.

Placing pH microelectrodes inside cells undoubtedly causes some damage, but the membrane potential is measured and the damage can be assessed. The damage usually consists of a 'leak' around the electrodes. This leak rarely causes a large change in pH_i because intracellular buffering power is high and the pH gradient across the cell membrane is low. The extent to which the membrane potential reflects the amount of damage is, of course, dependent upon the input resistance of the cell. The damage to large cells appears less than that to small ones. However, in both small and large cells the damage, if sufficient, will lead to a large influx of other ions such as calcium and sodium. This physical damage to the integrity of the cell membrane has limited the used of pH-sensitive microelectrodes to large and robust cells and represents a constant source of anxiety to those using ion-sensitive microelectrodes. The leak around the pH-sensitive microelectrode can also cause another major problem. If for some reason the pH-sensitive

microelectrode fails to record the full membrane potential, a relatively common phenomenon, then the pH signal following subtraction of the full membrane potential, as measured with the second electrode, will not be an accurate measure of pH_i. The only way to test for this subtraction error is to hyperpolarise the cell, a manoeuvre which in most cells (but not glial) does not change pH_i.

pH-sensitive dyes

Early dyes

Following De Vries' (1871) observations of colour changes in beetroot cells, others extracted and chemically identified various coloured pH-sensitive dyes. Attempts to 'load' such dyes into cells date back to early work on protozoa (Metchnikoff, 1893) using particulate litmus. By 1906, dyes like neutral red were being used to stain living cells and then 'measure' pH_i. Dye injection and early forms of 'scrape-loading' were also in their infancy around 1920. However, by the 1930s the use of pH_i indicators was in decline. The problems associated with their use were serious: salt, metachromatic and protein errors made absolute measurements difficult. However, many used absorbance dyes such as phenol red to measure relative pH changes (for example Ahmed & Connor, 1980), with some success. The introduction of fluorescent dyes in the early 1980s began a new era for pH_i measurement.

Fluorescent dyes

Fluorescent pH-sensitive dyes appeared to overcome many of the previous problems of optical pH measurement. The ratiometric approach – that is, dual excitation or dual emission – allowed for the simple correction for changes in path length, indicator concentration, leakage or photobleaching. There are now several different classes of pH-sensitive fluorescent dyes: fluoresceins, benzoxanthenes, rhodols and pyrenes. Each class of dye has a range of different properties (pK, excitation and emission wavelengths, lipid solubility, photostability, esterified forms and dynamic range), making them suitable for varying applications. However, since its introduction by Tsien in the early 1980s, 2′,7′-bis(carboxyethyl)-5(and 6)-carboxyfluoresceine (BCECF) has been by far the most popular. The fluorescent dye could be incorporated into cells by simply bathing them in the lipophilic acetoxymethyl (AM) ester form of the dye (BCECF-AM). The dye enters the cell where native esterases hydrolyse it, releasing the charged membrane impermeant BCECF. This allowed for pH_i measurements in even the smallest

of cells. It was recognised fairly early on that the probe could not be calibrated satisfactorily *in vitro* (for example, Chaillet & Boron, 1985). The antibiotic nigericin, however, proved to be a simple solution. Nigericin is lipid soluble and is easily incorporated into the cell membrane, where it exchanges protons for potassium. By altering the extracellular potassium concentration and pH, whilst knowing the intracellular potassium concentration, the pH_i can be set and an *in vivo* calibration obtained. The AM loading of BCECF and the simple nigericin calibration resulted in an explosion of work on pH_i. However, serious problems remained with the technique that are only now starting to be appreciated. The remainder of this chapter is focused on three particular problems: cell health, inhibition of transporters, and contamination with exogenous transporters.

Recent problems with dyes

Assessing cell health

Electrode measurement of pH_i necessitates measuring the cell membrane potential – this allows for an obvious and simple way of judging cell health. A loss of membrane integrity usually results in a profound depolarisation. The measurement of pH_i with fluorescent dyes provides no such information; cell health must instead be assessed visually. Most of the fluorescence microscopes available in the 1980s and early 1990s did not allow for the continuous visualisation of cells whilst recording pH_i. Now, however, cheap, charged-coupled devices are making long wavelength recording of images increasingly common.

Since pH_i and extracellular pH (pH_e) are similar (approximately 7.2 and 7.4 respectively) it is not possible to use pH_i as a discriminator of cell health. A near-complete collapse of the membrane potential as a result of a loss in membrane integrity, and therefore a Nernstian distribution of H^+, can result in little change in pH_i. However, one can use the dye signal to provide some information about membrane integrity. Schwiening and Boron (1992) and later Bevensee, Schwiening and Boron (1995) showed that the rate constant for dye loss, as assessed by the pH-insensitive fluorescence of BCECF, could be used as an indicator of cell health. Figure 5 shows records of pH_i, intracellular BCECF concentration and the rate of dye loss. A simple interpretation made by Schwiening and Boron (1992) was that the loss of dye in Figure 5, and therefore the profound alkalinisation, resulted from a loss in membrane integrity.

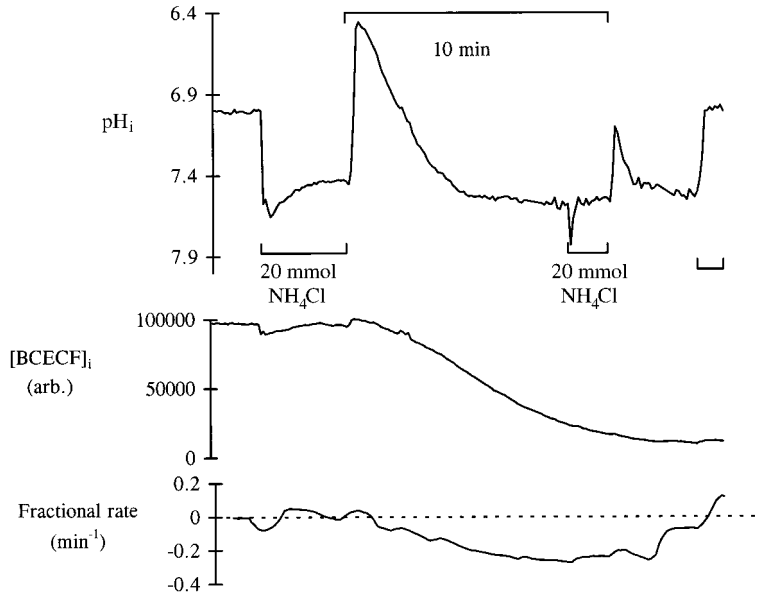

Fig. 5. pH_i, BCECF concentration (440 nm excited fluorescence) and the rate of dye loss measured in a rat hippocampal neuron. NH_4Cl application initially produced normal pH_i changes. However, within five minutes, pH_i approached the pH_e (7.4). A second NH_4Cl application produced only very small, transient pH_i changes. A nigericin/high K^+ calibration to pH 7.00 is shown at the end of the experiment. Data modified from Schwiening and Boron (1992).

They proposed the use of the instantaneous rate of dye loss as an indicator of membrane integrity. The technique is not entirely straightforward because some BCECF is tightly bound to cellular proteins and is not lost during a modest loss of cell integrity.

It is remarkable that so few choose routinely to show such data. For instance, Figure 6 is a recording of pH_i made by Hays and Alpern (1991) in the kidney, showing a striking spontaneous change in pH_i. This result was interpreted as the delayed activation of proton pumps. It is noticeable that the signal-to-noise ratio in the experiment increased during the alkalinisation – a common result of a loss of dye. It is surprising that the delayed activation of H^+ pumps in Figure 6 and the loss of cell integrity in Figure 5 can result in such superficially similar pH_i changes.

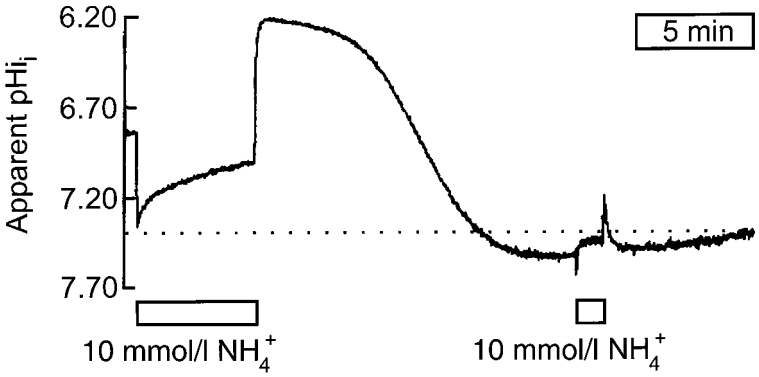

Fig. 6. pH_i measured with BCECF in renal outer medullary collecting duct cells. NH_4Cl application initially produced normal pH_i changes. After about ten minutes, pH_i spontaneously approached the pH_e, indicated by the dotted line. A second NH_4Cl application produced only very small, transient pH_i changes. Modified from Hays and Alpern (1991).

Inhibition of transporters

Free BCECF is highly charged, and hence retained within the cytosol. Unfortunately, this high charge causes the binding of BCECF to cellular proteins resulting in a large spectral shift of the dye. For this reason, the *in vitro* calibration cannot reliably be applied to *in vivo* measurement. Furthermore, one of the proteins to which BCECF binds is the calcium-ATPase. The inhibition of the calcium-ATPase by BCECF and other pH-sensitive dyes (see Table 1) was first demonstrated by Gatto and Milanick (1993) in red blood cells. This may at first sight seem of little consequence to those measuring pH_i. However, in 1993 Schwiening, Kennedy and Thomas showed that the only calcium extrusion mechanism in snail neurons, the calcium-ATPase, counter-transported H^+. It is thus possible that the calcium-ATPase or, more accurately, the calcium–hydrogen pump, is a major acid-loading mechanism. Measuring pH_i with BCECF is clearly fraught with possible distortions. Gatto and Milanick (1993) reported different IC_{50} values for BCECF obtained from different suppliers. Nevertheless, many of the pH_i measurements made with BCECF may have been done in systems lacking what could be a major acid-loading mechanism, especially during calcium loading that follows electrical activity.

Fortunately, there are pH-sensitive dyes available that do not inhibit

Table 1. *Various inhibitors, including pH-sensitive dyes, and their IC_{50} concentrations for the calcium pump in red blood cells*

Compound	IC_{50} (μM)
Fluorescein	1000
DM-NERF	500
BCECF	100
5,6-Carboxy-SNAFL-1	65
Eosin B	0.05

Data from Gatto and Milanick (1993).

the calcium pump, for example 8-hydroxypyrene-1,3,6-trisulphonic acid (HPTS). Figure 7 shows acid pH_i transients caused by the counter-transport of H^+ on the calcium pump following depolarisation. HPTS was loaded into the neuron through the patch-pipette. Figure 7 also shows the inhibition of the calcium pump by eosin (see Table 1). HPTS provides a good signal-to-noise ratio when compared to BCECF, especially at acidic pH (less than about 7.2). The major drawback of HPTS is that, unlike BCECF, it is not available in a membrane-permeant form.

Contamination with ionophores

The nigericin technique is almost universally used to calibrate pH-sensitive dyes. However, it has a potentially very serious drawback. Richmond and Vaughan-Jones (1993) showed that when nigericin, a potassium–hydrogen exchanger, was used for pH_i calibration it could subsequently contaminate cells used in the same apparatus, even several days later. This contamination occurred even though the bath and tubing had been thoroughly rinsed. The result of the contamination was a marked acid loading and profound pH_i changes on altering external potassium. Wilding, Cheng and Roos (1992) had shown exactly these effects and concluded that rat carotid body possessed a native potassium–hydrogen exchanger. Richmond and Vaughan-Jones (1993), working on the same cell type, showed that washing the experimental apparatus for several days with 20% Decon removed the nigericin contamination. They concluded that carotid body type-1 cells showed no native potassium–hydrogen exchanger.

It is likely that many of the researchers using nigericin to calibrate pH-sensitive dyes contaminated their cells with some molecules of

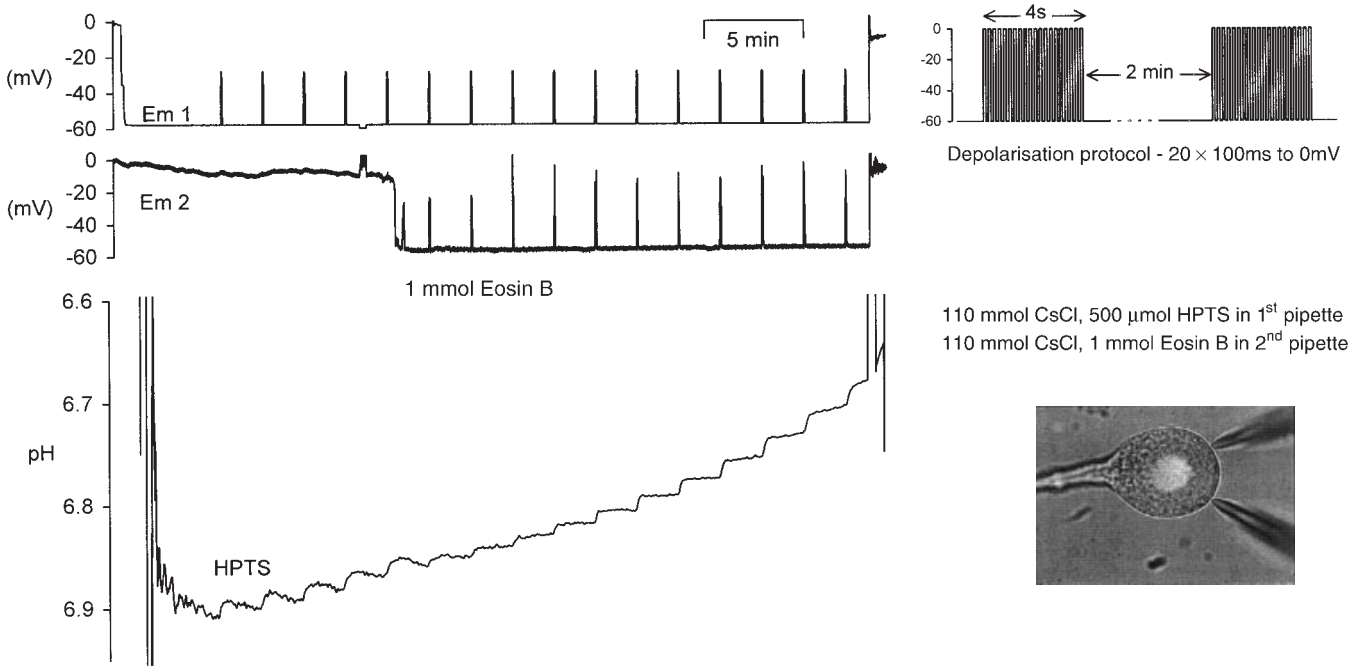

Fig. 7. pH_i measured in an isolated snail neuron (∅ ~50 µm) using HPTS during depolarisation. The first patch-pipette (Em 1) was used to introduce HPTS. The second patch-pipette (Em 2) was used to introduce eosin B. Modified from Schwiening (1997).

exogenous potassium–hydrogen exchanger. There are alternative methods for calibrating pH_i. In cells too small to be impaled with a pH-sensitive microelectrode, the weak acid–base method (Szatkowski & Thomas, 1986; Eisner *et al*., 1989) can be used. This technique involves the application of a weak acid and base in proportions that result in no pH_i change. The pH_i can be calculated from the proportions of the weak acid and base. This technique is cumbersome, and only results in a one-point calibration; however, it avoids any possibility of nigericin contamination. pH-sensitive dyes may also be calibrated *in vitro* but solutions may need to mimic the intracellular environment (e.g. high potassium, low calcium and include ATP and proteins).

Highs and lows of dyes and electrodes

Having presented an account of some serious problems associated with fluorescent pH-sensitive dyes it would be wrong to leave the reader with such negative feelings about them. Table 2 shows a comparison between dyes and electrodes. It can be seen that dyes are easy to load into cells, causing little damage, require minimal manual dexterity and can be used on commonly available inverted microscopes. Dyes can be used to monitor pH_i in several cells at once, even moving ones, and records are not complicated by electrical artefacts. If one can assess the problems (uncertain calibration, possible nigericin contamination, lack of membrane potential measurement, limited pH range and inhibition of transporters), then dyes remain the method of choice.

The disadvantages of pH-sensitive electrodes are clear in Table 2, the main one being damage. Most workers measuring pH_i in mammalian non-muscle cells would find it very difficult to make pH-sensitive microelectrodes small enough to use, and it is likely that dyes will remain the method of choice for them. However, pH-sensitive microelectrodes are easy to calibrate, stable and can be used on electrophysiological rigs with minimal extra expense.

Recommendations for further reading

Although now slightly dated, Thomas (1978) can be recommended for more details on using microelectrodes to measure pH. Ammann (1986) contains much information on pH-sensitive ligands, whilst Purves (1981) discusses some of the wider issues relating to microelectrode use. *Microelectrode techniques: The Plymouth Workshop Handbook* (Ogden, 1994) also contains useful methodological nuggets of wisdom. Finally, this author always enjoys a trawl through the latest *Molecular*

Table 2. *Comparison of pH-sensitive microelectrodes and fluorophores*

	pH-sensitive microelectrodes	Fluorescent dyes
What is measured?	Nernstian for H^+ across a membrane	Buffering of H^+ causing a change in fluorescence
Where is pH_i measured?	Point source defined by electrode tip location	Diffuse volume dependent on optics and system
Can pH_i be measured at multiple locations simultaneously?	No	Yes, depending upon system
Cost of equipment	Low	High but many off-the-shelf products
Probe fabrication	Either difficult to make or temperamental	Available off the shelf
Reproducibility of probe characteristics	Low	High
Ease of use	Requires manual dexterity	Mostly trivial
Insertion of probe	Requires two barrels to be inserted into cell	By diffusion (AM loading across membrane or free acid through patch-pipette)
Damage caused by probe	Physical to membrane	Inhibition of transporters and possible toxicity of dye or by-products
Factors which disrupt signal	Electrical activity, fluctuations in temperature or CO_2	Various drugs, for example harmaline and caffeine
Response speed	Slow ($\sim$1 s)	Fast
Linearity of response	Very good over wide range	Limited linear range
Calibration technique	In vitro	Nigericin/high K^+ or weak acid and bases
Calibration accuracy	High	Relatively uncertain
Calibration problems	None	Nigericin contamination
Resolution	Good	Poor
Size of cells	$>\sim$50 μm	Any size
Need to visualise individual cells?	Yes, unless working on electrically tightly coupled cells	No
Can pH_i be measured during cell movement?	No, absolute requirement for stability	Yes, depending upon system

probes handbook (http://www.probes.com/) and the *Fluka selectophore* catalogue.

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