QUANTITATIVE PRESSURE INJECTION OF PICOLITER VOLUMES INTO *LIMULUS* VENTRAL PHOTORECEPTORS

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ABSTRACT Many pharmacological probes must be applied to the interior of cells to produce their effects. Ideally, a method for injecting such materials should be simple, rapid, and independent of the chemical properties of the material to be injected. In addition, one might desire to confirm immediately that an injection occurred and to estimate the volume injected shortly thereafter. We report that these conditions are fulfilled when the injection of materials from micropipettes by pressure pulses is confirmed by visualization of injection-induced disturbances in cells viewed on a video monitor. Volumes of aqueous droplets subsequently injected into a nearby oil pool may be used to estimate the volumes injected into cells. We have obtained a calibration curve for these quantitative estimates of injected volumes by injecting radioactively labeled sulfate into *Limulus* photoreceptor cells. We find that the estimates are accurate within a range covering one order of magnitude. We assess the sources of systematic and random errors in making these estimates.

INTRODUCTION

As part of a study of visual excitation, we have examined the intracellular effects of a variety of phosphatase inhibitors in *Limulus* ventral photoreceptors (Fein and Corson, 1979; 1981). These compounds were injected into the cells by the method of ionophoresis. Recently we adopted the method of pulsed-pressure injection so that we could deliver compounds into the photoreceptors without regard for their individual ionophoretic mobilities (see Purves, 1980, for a discussion of the limitations of ionophoresis). In the course of our experiments, we discovered that we could immediately confirm the occurrence of an intracellular injection independently of the effects of the compounds in the pipette if we watched for local disturbances in the cytoplasm on the video monitor of an infrared viewing system while we applied pressure pulses to the back of the pipette. As these observations have greatly improved the utility of the pressure injection method in our hands, we illustrate the video confirmation of injections in this report.

Having a reliable means for confirming the success of an injection, we went on to develop a procedure for estimating the volume of solution injected into a cell. Our procedure is to inject material into a cell using a series of pressure pulses and then estimate the volume injected by subsequently measuring the size of a droplet of solution ejected from the pipette into a nearby pool of vegetable oil by an identical series of pressure pulses. We chose this method because we wished to have an immediate estimate of the volume delivered and because we wanted to avoid the constant use of radioactive contaminants for measuring the injected volume of experimental solutions. In the present experiments, we have included radioactively labeled sulfate in the injection solutions, and thereby obtained a calibration curve for our estimates of volumes injected into cells. As will be explained in the text, the injection procedure and calibration curve allow us to estimate the volume delivered within a range covering one order of magnitude.

Some components of the procedure we use have been employed previously for purposes other than the present calibration of estimates of volumes injected into cells. Both the basic apparatus for pulsed injections and the visualization of ejected volumes in oil have been described earlier for extracellular applications of pharmacological agents (McCaman et al., 1977; Sakai et al., 1979). Radioactively labeled sulfate has previously been used to determine the volumes of experimental solutions injected into *Limulus* ventral photoreceptors (Brown and Blinks, 1974; Lisman and Brown, 1975; Coles and Brown, 1976). We have combined these components to calibrate the procedure for estimating volumes injected into cells. We have also used this combination of methods to evaluate the sources of error in making these estimates.

METHODS

Pressure pulses of ~20 psi (range 10–30 psi) and ~300 ms duration (range 100–600 ms) were applied to the back of micropipettes by means

of a self-relieving electropneumatic valve (model EV-3-12V; Clippard Instrument Laboratory, Inc., Cincinnati, OH). Pressure at the valve was set by a low pressure regulator (model MAR-1; Clippard Instruments Laboratory, Inc.) and monitored with a pressure gauge (model PG-100; Clippard Instruments Laboratory, Inc.). The source of pressure for the valve was a nitrogen cylinder with a conventional two-stage regulator.

Pipettes for pressure injection were prepared from 1-mm borosilicate thin-wall glass tubes with capillary fibers (model 30-30-0; Frederick Haer & Co., Inc., Brunswick, ME). These were pulled on a horizontal Brown-Flaming electrode puller (model P-77; Sutter Instruments Co., San Francisco, CA). Pipettes were mounted in an electrode holder (model MEH-2S-1.0; W-P Instruments, Inc., New Haven, CT) containing a silver/silver chloride pellet for electrical contact and a pressure port attached by ordinary 1.27-mm polyethelene tubing (Intramedic No. 7421; Clay Adams, Parsippany, NJ) to the electropneumatic valve.

Conventional methods, which have been described in detail elsewhere (Fein and Charlton, 1975; 1977), were used for impaling and recording from the ventral photoreceptors of *Limulus polyphemus*. Potassium aspartate (Lisman et al., 1979) was included in the injection solutions to lower the resistance of the pipettes. Cells were injected under infrared illumination provided by a 45-W tungsten-halogen lamp (6.6A/T2 1/2 Q/C1; Sylvania Consumer Lighting, Danvers, MA) and an infrared filter (model RG-1000; Schott Optical Glass, Inc., Duryea, PA). A microscope formed an image of the cell upon a video camera (model WV-1350A; Panasonic Co., Div. of Matsushita Electric Corp. of America, Secaucus, NJ) having an extended red sensitivity tube (Newvicon type S4113; Panasonic Co., Div. of Matsushita Electric Corp. of America). Contrast of the video image was enhanced by a video processor (model 604; Colorado Video, Inc., Boulder, CO). Images of cells during injections were stored on the video disk of a motion analyzer (model SVM-1010; Sony Corp. of America, Long Island City, NY) for frame by frame viewing and photography.

We included radioactive material in our injection solutions so that we could objectively determine the volume of material injected into cells. The injection solutions contained 25 mM $Na_2\ ^{35}SO_4$ (25 mCi/ml; New England Nuclear, Boston, MA) and 91 mM KAspartate. Injected cells on nerves pinned to clear elastomer blocks (Sylgard; Dow Corning Corporation, Midland, MI) were removed from the artificial sea water (ASW) (Fein and Corson, 1981) bath, rinsed once by immersion in a second bath of ASW, and transferred to a scintillation vial. Labeled sulphate was released from the cells by heating them to 75°C for 10 min in a mixture of 0.2 ml of 60% perchloric acid and 0.4 ml of 30% hydrogen peroxide (Mahin and Lofberg, 1966). Following release of the labeled sulphate, the samples were cooled, mixed with 9 ml of Aquasol scintillation cocktail (New England Nuclear) and allowed to stand for 1 h before counting. Volumes of solutions injected into cells were calculated from the counts of radioactivity made on a scintillation counter (model 3330; Packard Instruments Co., Inc., Downers Grove, IL).

The accuracy of dilution of the radioactive standards used to determine isotopic volumes is on the order of a few percent and was limited by the accuracy of the pipettor (0.8%, Pipetteman models P-200D, P-1000D; Rainin Instrument Co. Inc., Woburn, MA) used for the dilutions. We assess the random error of measurements of recovered isotopic volumes to be on the order of 10% of the determined value based on the expected statistical fluctuations in the counts (typically, for each sample, we counted a few hundred to a few thousand scintillations). Previous work by Coles and Brown (1976) sets a probable upper limit of 20% on the volume of sulfate lost by leakage from the cell before recovery of the sample.

For calibration of the estimates of injected volume, data were fitted to a conventional logarithmic regression model given by Eq. 1

$$y_i = a_0 + a_1 x_i + \epsilon_i, \tag{1}$$

where a_0 and a_1 are the intercept and slope parameters for the regression; ϵ_i is the error term, and y_i and x_i are the natural logarithms of isotopic and geometric volumes, respectively. Our reasons for choosing the logarithmic transform of the variables will be given in the Results section. The model was used to plot the regression line in Fig. 3, which gives the least-square estimates of isotopic volumes from geometric volumes.

The precision of our geometric estimates of injected volumes is given by the accompanying confidence intervals (Fig. 3). For a criterion level, (α = 20%), a ($100 - \alpha$) confidence interval (Afifi and Azen, 1972; p. 96) for an individual estimate ($\hat{y}$) of the log isotopic volume (y) at a log geometric volume (x) is given by

$$\hat{y} \pm st \cdot [1 + 1/N + (x - \bar{x})^2 / \Sigma_{i-1}^{N} (x_i - \bar{x})^2]^{1/2}.$$

In the expression above, s is the standard error of the estimate, $\bar{x}$ is the mean, and t is the value of student's t distribution at a criterion level of $(100 - \alpha/2)$ for $N - 2$ degrees of freedom, where N is the total number of data points in the regression.

To assess the sources of error in our estimate of volumes injected into cells, we have chosen measures of systematic and random errors that are expressed on the original scale of picoliters. Our measure of systematic error is the ratio of the geometric mean of the isotopic volume to the geometric mean of the geometric volume. These geometric means (in picoliters) were obtained from the simple antilog transforms ($e^{\bar{x}}$, $e^{\bar{y}}$) of the arithmetic means of the log volumes. Our measure of the extent of random error about the geometric mean of the isotopic volume is given by the picoliter range covered by one conditional standard deviation of the sample of log isotopic volumes using the transformation given below (Aitchinson and Brown 1969)

$$e^{\bar{y} \pm S_c}.$$

The conditional standard deviation S_c above is a measure of uncorrelated random errors in a regression and is given by Eq. 2

$$S_c = [S_y^2 (1 - r^2)]^{1/2}, \tag{2}$$

where r is the correlation coefficient, and S_y^2 is the variance of the log isotopic volumes (Afifi and Azen, 1972). Assuming that the random errors are normally distributed in the log domain, which we have checked experimentally (see Results), the antilog transform used above preserves information on the quantiles of the distribution in the linear domain (Aitchison and Brown, 1969).

RESULTS

We found that we could reliably inject materials into cells if we first confirmed that the pipettes would eject droplets of aqueous solution into vegetable oil. To prepare a pipette for injection, the tip was broken incrementally under the microscope by pushing it against the shaft of a second pipette held at approximately right angles to the injection pipette. This procedure was repeated until the electrode would reproducibly eject aqueous droplets into the oil. When a pipette became blocked during intracellular injection, we could sometimes overcome the blockage by raising the pressure of successive pulses until the blockage cleared. The procedure for breaking pipette tips could be repeated to conveniently restore a pipette that had become clogged after several impalements. Examination of the injection pipettes under a microscope with a 100× objective indicated that the tips were on the order of 1 μm or less in diameter. With these electrodes, a pressure pulse of 10–30 psi and 100–600 ms duration typically delivered less than 10 pl (picoliters) of solution into the oil.

Video Confirmation of Injection

Two features of successful intracellular injections were visible on the video monitor, but these frequently occurred at separate planes of focus under the microscope. Two typical observations in separate focal planes are illustrated in Fig. 1. First, a local disturbance can be seen in focal plane 1 (part *B*) near the center of the cell and below the plane where the electrode enters the cell. We interpret this disturbance to be a displacement of organelles and a portion of the cell membrane. Second, a diffuse local darkening in the region of the pipette tip can be seen higher up in focal plane 2. This darkening is probably associated with local changes in the refractive index as the bolus of material from the pipette enters the cytoplasm. Similar diffuse changes in transmitted intensity could be seen during injections into ASW. In a given injection, one or both of the features shown in Fig. 1 may be visible on the video monitor. We wish to emphasize that the changes illustrated in Fig. 1 appear much more pronounced when temporal information is available on the video screen during actual injections.

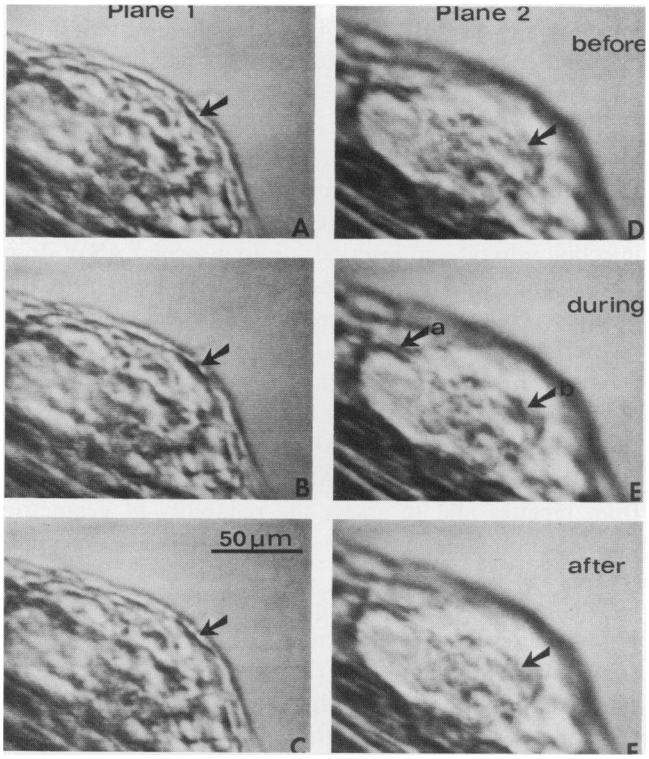

FIGURE 1 Representative video frames illustrating the two features commonly observed when a cell is injected. The left column shows what appears to be a local displacement of organelles at one plane of focus during an injection (*Plane 1*). In *A* the arrow points to a concave region of the cell membrane before injection. The local area of membrane becomes convex during the injection shown in *B* and then relaxes back in *C*. The right column shows a diffuse local darkening (*b, frame E*) of the region around the electrode tip during an injection viewed at a higher focal plane (*Plane 2*) in the cell. Part of the shaft of the injection electrode (*a, frame E*) is visible in the frames of the right column.

Throughout the series of injections we monitored the condition of the cells both before and after injection. All of the cells used in this study had receptor potentials of at least 30 mV in response to a 20-ms test flash attenuated 1.5 log units from the maximum available intensity. These potentials appeared similar to those normally obtained with conventional KCl-filled pipettes. Large injections sometimes caused a temporary desensitization of the cells that lasted for a few minutes and was followed by a recovery of sensitivity to roughly the level observed before injection. By these criteria, the cells did not appear to be unduly damaged by penetration with the injection pipettes or by the injections themselves.

Calibration of Injections into Cells

Having a reliable method for confirming the success of injections into cells, we went on to obtain a quantitative estimate of the volume of material injected. After a successful injection of radioactively labeled sulfate by a series of pressure pulses (typically 4 or 5), we withdrew the electrode and inserted it into a nearby pool of oil on the bottom of the recording chamber. We then applied an identical series of pressure pulses that ejected a droplet of aqueous solution (Fig. 2) whose volume we calculated from the average of two perpendicular measurements of its diameter. Of the 45 cells used in this study, 34 were injected by this method using 15 separate electrodes. Leakage from electrodes was estimated in the remaining 11 cells.

In Fig. 3, we have plotted isotopically determined volumes against the corresponding geometric measurements of droplets injected into oil. From the data in Fig. 3, a calibration curve for the estimation of isotopic volumes from the geometric volumes was obtained by standard least-squares regression. We chose to calculate the regression line from the natural logarithms of the volumes rather than the volumes themselves because the logarithmic transformation results in a larger coefficient of correlation

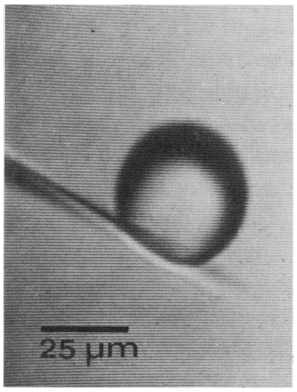

FIGURE 2 A droplet of aqueous solution ejected from a pipette immersed in vegetable oil. The volume of the droplet produced by four pressure pulses (20 psi, 400 ms) is estimated to be 27 pl.

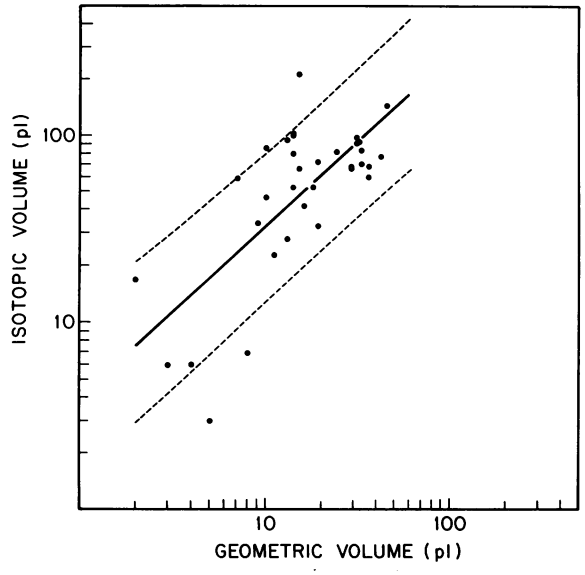

FIGURE 3 Isotopic calibration of geometric estimates of volumes injected into cells. The data points are isotopically determined volumes of labeled sulphate solution recovered from cells plotted against geometric estimates made from the diameters of droplets ejected into oil following successful injections into cells. The straight solid line (—) represents the least-squares regression line of the log isotopic volume on the log geometric volume. The curved dashed lines (---) represent the limits of the 80% confidence interval for estimates of an individual isotopic volume from an individual geometric volume (see Methods). Values of regression parameters for this experiment are: $a_0 = 1.40$, $a_1 = 0.91$, $r = 0.71$, $N = 34$, $S_c = 0.28$, and $S_e = 0.69$. The geometric mean of the isotopic volumes was 48 pl. The value of the correlation coefficient for regression in the linear domain was 0.47.

and hence a stronger relation between isotopic and geometric volumes. The log regression model is preferred for this reason and because errors in the estimate of geometric volume are governed by the cube of errors in measurement of the diameter (see Gaddum, 1945) and hence are not independent of the injected volume as would be assumed in a linear regression of the untransformed volumes. We also expect that variation in the volume injected into cells is unlikely to be independent of the absolute size of the injected volume.

From Fig. 3 we find that injection into cells delivers on average 48 pl, a systematic error about three times the value estimated simply from the geometric mean of the geometric volumes of subsequent injections into oil (15 pl). The calibration curve allows us to correct for this systematic error in the accuracy of the estimate. The average injected volume of 48 pl determined by isotopic recovery amounts to ~10% of the average cell volume. Of the 45 cells used in this study, cell volumes for 38 were estimated by measuring their length and width and calculating the volume of an equivalent prolate ellipsoid. The mean and standard deviation for estimated cell volumes used in this study was 440 ± 184 pl. Visualization of the remaining seven cells was not clear enough for measurement of their dimensions.

To establish the precision of our estimate of injected volume we calculated a confidence interval (see Methods) about the regression line over the working range of our injections. From Fig. 3 we find that the half confidence interval on one side is threefold greater than and on the other side is threefold less than the estimated volume over the range of estimates. Since systematic errors are eliminated by the calibration and since the confidence interval covers a range corresponding to about one logarithmic unit, the accuracy of the estimate is within one order of magnitude surrounding the estimate.

Sources of Error in the Estimate

Having established a method for estimating the injected volume, we sought to examine the sources of systematic and random error. To this end, we replicated the injection procedure and regression analysis used for cells on a series of small pools of oil and drops of ASW where we could recover the labeled sulfate from both the oil and the ASW. The sequence of injections was as follows. We first injected into a pool of oil to provide a direct isotopic measure of volumes injected into oil (DROPS, Fig. 4) and to establish a basis for examining the reproducibility of a second injection into oil (OIL, Fig. 4). To mimic injections into a cell we then applied an identical series of pressure pulses to inject labeled sulfate into a nearby droplet of ASW (ASW, Fig. 4). Next a second oil pool was injected by a third set of identical pressure pulses to provide a geometric estimate of the volumes previously injected into oil and into ASW. Sometimes we withdrew the electrode after the second injection into oil and inserted it into a second ASW drop for 10 min to determine the rate of leakage from the pipettes. Following the injections, labeled sulfate was recovered from the pools of oil to determine the error in the geometric measurements of the volume of aqueous droplets of oil (DROPS, Fig. 4) and to assess the reproducibility of successive injections into oil (OIL, Fig. 4). Labeled sulfate was also recovered from the ASW to determine the leakage from pipettes and to assess the reliability of geometric estimates of injections into a simple aqueous medium (ASW, Fig. 4).

The net results of our error analysis are summarized in Fig. 4 where the geometric means of the isotopic volumes (first three clear bars) and the extent of the conditional standard deviations in picoliters (interval markers) are compared with the geometric means of the geometric volumes (striped bars) for each of the three conditions described above. For comparison, the calculated regression estimate of isotopic volume (fourth clear bar) and extent of the conditional standard deviation for hypothetical 25-pl injection into cells (Fig. 3) are shown in the fourth column of Fig. 4. We tested the distributions of the residuals from the four log regressions used to calculate the conditional standard deviations represented in Fig. 4. Based on the nonsignificant ($p > 0.05$) values of the four chi-square statistics for goodness of fit, we accept the null hypotheses

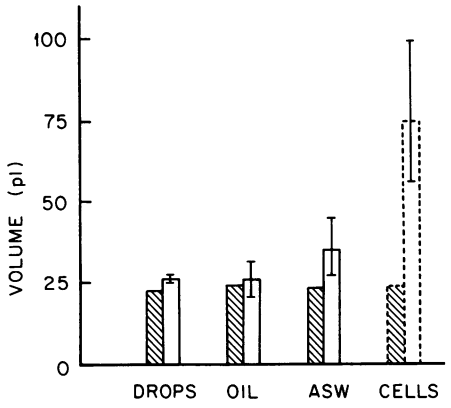

FIGURE 4 Systematic and random deviations of isotopic volumes (clear bars and interval markers) from corresponding geometric volumes (striped bars) under four different injection conditions described in the text. The first three pairs of bars are the result of 26 sequences of injections into oil pools and drops of ASW using 11 separate electrodes. The heights of the oil and ASW geometric volume bars differ slightly because two preliminary injections into oil were lost.

that the residuals from the log regressions are normally distributed, and we conclude that we may use the antilog transform (see Methods) to calculate the corresponding extent (in picoliters) of the conditional standard deviations about the geometric means of the isotopic volumes as given in Fig. 4.

The first pair of bars in Fig. 4 (DROPS) shows that there is good agreement between isotopic and geometric volumes measured in the same drop, and that the random error is small despite the sometimes nonuniform shape of droplets and the limited resolution of the edges (Fig. 2). The 50 droplets used in these measurements were delivered by a total of 11 separate pipettes that were also used for concurrent injections into ASW. The second pair of bars in Fig. 4 (OIL) shows that there is little systematic error in estimating the isotopic volume of one injection into oil from the geometric volume of a later injection into another oil pool. As expected, there does appear to be an increase in the random error over that encountered in measuring both in the same pool of oil (DROPS, Fig. 4). These measurements demonstrate that repeated injections within one medium are reasonably reproducible. The third pair of bars in Fig. 4 (ASW) shows that an apparent systematic error arises in the estimation of isotopic volumes injected into an aqueous medium and that random errors increase as well. For comparison, the calculated systematic and random errors associated with 25-pl injections into cells are given in the fourth pair of bars in Fig. 4 (CELLS). Again, there appears to be an increase in both systematic and random errors.

As errors in the isotopic volumes may arise due to leakage from electrodes, 11 cells were impaled with injection pipettes for 10 min each, but no pressure pulses were applied. After impalement of the cells, the competence of each pipette for injections was confirmed by injections into oil. The simple arithmetic mean and standard deviation of the equivalent isotopic volume of material that leaked into the cells was 3.4 ± 2.0 pl. Cells that were injected for the measurements in Fig. 3 were generally impaled for no longer than 2 to 3 min. Therefore, the contribution of systematic errors due to leakage from the pipette to the average injected volume of 48 pl was probably <10% of the injected volume. Similar values for leakage (3.1 ± 3.8 pl) were found for 16 comparable measurements of leakage into drops of ASW from 7 electrodes.

DISCUSSION

The method described here for injection has already proven to be quite useful in the delivery of a variety of compounds, including proteins, into cells. The advantages of the method are (*a*) the immediate confirmation of delivery, (*b*) the reliability of injections into a sequence of cells, (*c*) the recovery of plugged electrodes, (*d*) the absence of stringent requirements for the filtration of injection solutions (we typically do not filter our solution), (*e*) the freedom from constant use of radioactive contaminants as a measure of injected volume, and (*f*) the prompt generation of quantitative estimates of injected volumes.

We have used the volumes of aqueous droplets injected into oil to estimate the volumes injected into cells. As the calibration procedure compensates for systematic errors, the accuracy of the method is determined by the range of random error in the estimate. We find that the range of error in our procedure covers one order of magnitude around the estimate (see Results).

Sources of Error in the Estimate

Systematic errors in the estimation of injected volume arise primarily from factors associated with injections into cells and from differences in the forces of surface tension acting at the tip of the electrode when injections are made into oil vs. an aqueous medium (Fig. 4). In oil the surface tension opposes the injections, whereas in an aqueous medium diffusion and a net outward hydrostatic pressure prevail. The larger systematic error found for injections into cells indicates that either contact of the electrode tip with cell membranes or subtle changes in technique during injections into cells also contribute to systematic errors in the estimate. Leakage from electrodes appears to make a minor contribution to systematic error.

As gauged by the conditional standard deviations in Fig. 4, random errors in the estimate appear to increase on going from oil to water to cells. It may be possible to improve the accuracy of this method of estimation either by altering the pressure pulses or electrode tips so as to reduce the effects of surface tension when injecting into oil or else by identifying the additional sources of error introduced into cells. However, further experiments would be required to explore these possibilities, and we have found the present method to be satisfactory for our immediate purposes.

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