Journal of Biochemical and Biophysical Methods, 22 (1991) 243–252

ADONIS 0165022X9100066D

JBBM 00873

Microinjection into *Xenopus* oocytes: a precise semi-automatic instrument and optimal parameters for injection of mRNAs

Gabor Tigyi and Ian Parker

Laboratory of Cellular and Molecular Neurobiology, Department of Psychobiology, University of California, Irvine, CA, U.S.A.

(Received 16 July 1990)
(Accepted 26 November 1990)

Summary

A new apparatus for the injection of *Xenopus* oocytes is described which provides semi-automatic cell handling together with highly accurate and reproducible volume delivery. Using the system requires very little skill, yet it gives 6.3% average reproducibility in the 5 to 70 nl volume range. The instrument uses a fixed injector system driven by an Inchworm piezoelectric positioner or, in a low-cost version, by a Rainin EDP-2 battery-operated motorized pipette. A movable, vacuum-operated oocyte holder minimizes lateral movement of the oocyte during injection. Oocytes injected with the system show better survival and enhanced expression of mRNA compared with those injected with a widely used type of manual injector (Coleman, 1984).

Key words: Oocyte; mRNA; Translation, in vivo; Intracellular injection; Kainate-receptor

Introduction

Oocytes from the frog *Xenopus laevis* have become a widely used in vivo translation system for nucleic acids derived from tissues and from cloned genes [1–3]. For the neurobiologist, the oocyte expression system facilitates the electrophysiological study of functional voltage-activated ion channels and neurotransmitter receptors, as well as providing a method of screening for cloned genes

Correspondence address: G. Tigyi, Lab. of Cellular And Molecular Neurobiology, Dept. of Psychobiology, University of California Irvine, Irvine, CA 92717, U.S.A.
This work was supported by grants GM39831 and NS23284 from the U.S. Public Health Service.

encoding ion channels and receptors [4–6]. These experiments require controlled and accurate delivery of nanoliter volumes of nucleic acids into the oocyte in order to ensure consistent levels of expression. Most current injection systems [7–11] require considerable skill to obtain the necessary consistency and avoid damage to the oocyte. Even then, injecting large numbers of cells remains very tiresome. In this paper two versions of a new apparatus are described which provide highly accurate volume delivery with fast, semi-automatic cell handling and injection, and which are easily used by individuals with little or no experience in oocyte injection. This apparatus also ensures greater reproducibility compared to previously published methods [7–11] over a wide volume range. The low cost version, which uses parts of a commercially available digital motorized micropipette, although inexpensive, provides similar reproducibilities. Using this high precision instrument for the injection of radioactively-labeled tracers, leakage as a function of the total injected volume was measured and the optimal injection volume was also determined.

Materials and Methods

Materials used for construction of the injector

The injector was constructed from the following commercially available instruments: (1) Inchworm PZ550 piezoelectric positioner (Burleigh Instruments Inc., Burleigh Park, Fishers, NY), or in the low-cost version a Rainin EDP-2 battery-operated motorized pipette was used (Cat. No. E2-1000, list price US$245; Rainin Instruments Co. Inc., Emeryville, CA); (2) 10 μl gas-tight Hamilton syringe (Model No. 1701RN, Mfr. No. 80030); (3) Narishige micromanipulator (Model MM-3, Narishige Scientific Labs., Tokyo, Japan); (4) Stereomicroscope (Wild, Heerbrugg, Austria). Both the injector and the oocyte holder were attached to Yuasa (Yuasa International, Japan) magnetic bases and placed on a 1/4 inch-thick galvanized steel base-plate. For loading the mRNA, Eppendorf tubes containing nanoliter droplets were attached using plasticine to a Narishige (Model ML-8) micromanipulator, which allows the three-dimensional movement of the sample tube.

Construction of the instrument

A construction overview of the injector unit is shown in Fig. 1, while Fig. 2 shows the parts of the injector design.

The injector unit The moving arm of a programmable Inchworm piezoelectric positioner or the EDP-2 step-motor-controlled pipettor was attached to the plunger of a 10 μl gas-tight Hamilton syringe as shown in Figs. 2B and C. The digital micropipettor was disassembled and the controller electronics and battery remounted in a separate box, while the stepper motor was disconnected from the pipettor's original piston and attached to the syringe holder. A Hamilton syringe was clamped between two cylindrical aluminium pieces by a perpendicular tightening screw (arrow 5, Fig. 2B). The syringe plunger was connected by a screw cap (arrow 3, Fig. 2B) to the threaded end-piece fitted on the tip of the moving arm. This screw cap had a 1 mm radial slit to allow the placement and alignment of the

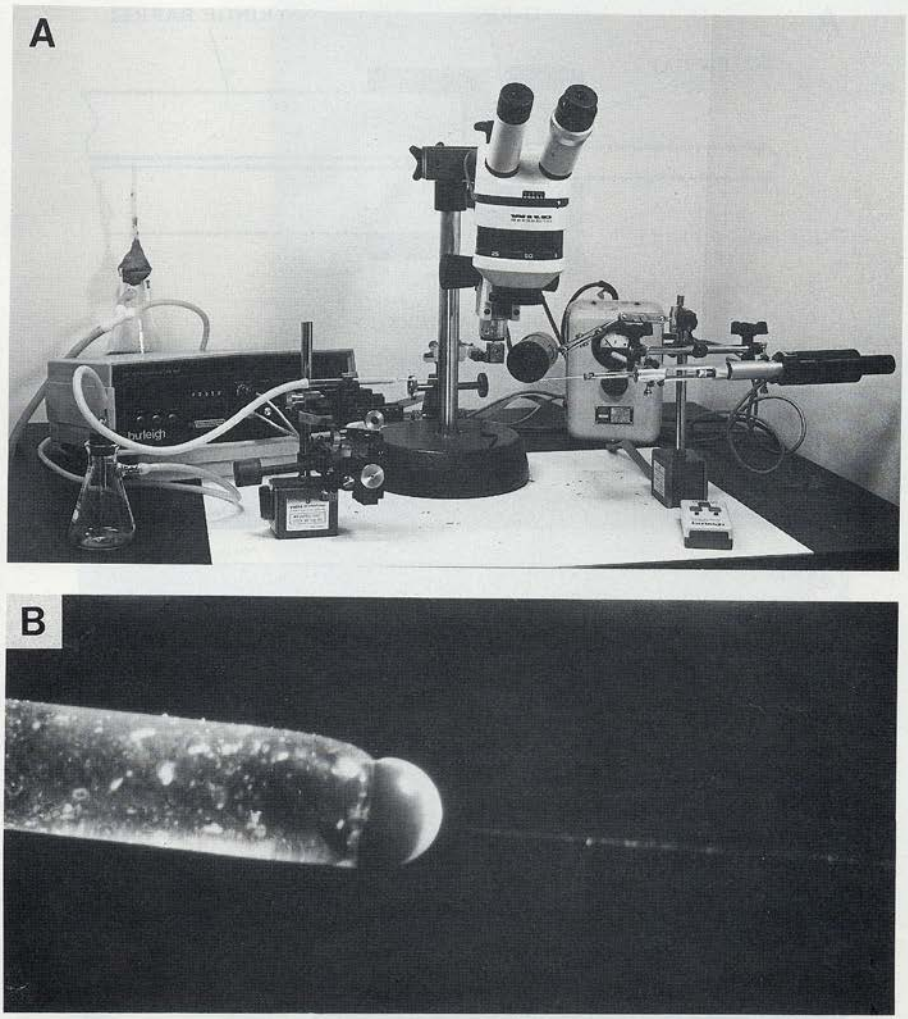

Fig. 1. A: complete injection system; B: oocyte held in suction pipette, with injection needle positioned ready to impale the cell. The diameter of the oocyte is approximately 1 mm.

syringe plunger with the axis of the moving arm. The machined aluminium syringe holder screwed onto the body of the Inchworm or the plastic housing of the stepping motor.

Mounting the injection pipette The method of mounting the glass injection pipette onto the syringe is shown in Fig. 2A. The removable needle of the syringe was discarded, and the screw cap drilled to form a hole of 2 mm diameter, sufficient to accommodate the injection pipette. The Teflon washers supplied with the syringe were cut into two equal length pieces and drilled to an internal diameter of 2 mm. A rubber O-ring (World Precision Instruments, New Haven, CT: outer diameter 3

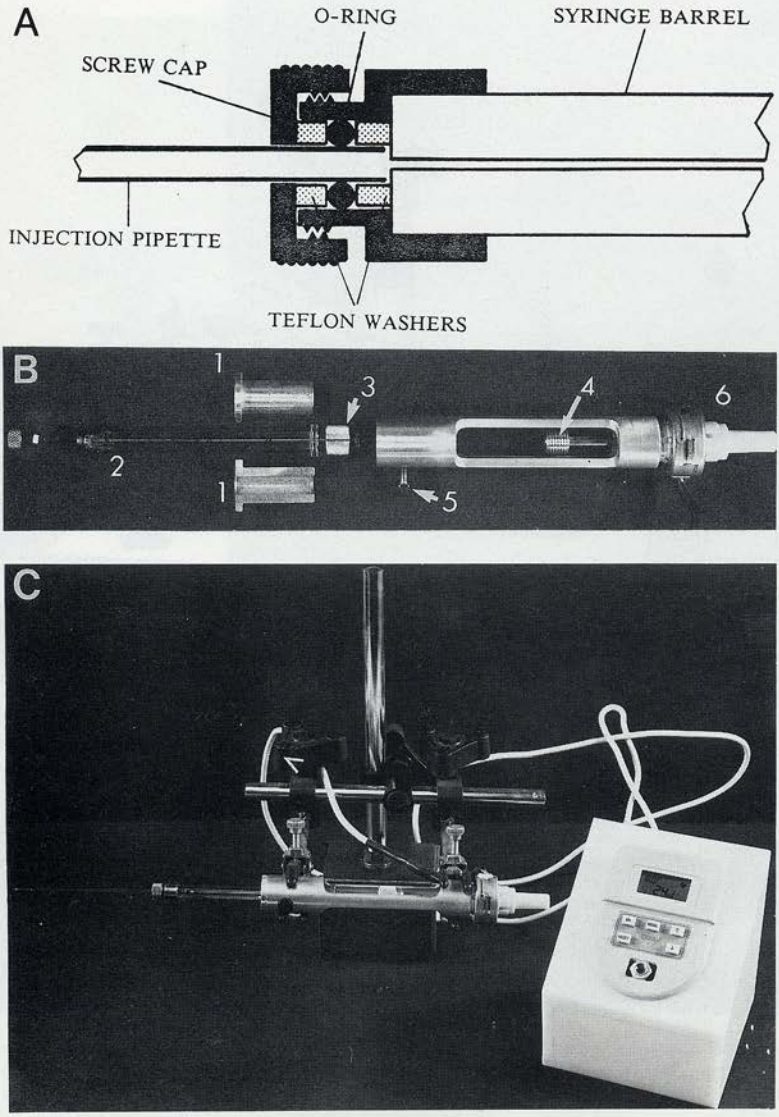

Fig. 2. Construction of the injector unit. A: modification of the Hamilton 10 μl syringe to allow mounting of glass injection pipette; B: components used to mount the syringe on the stepper motor: (1) syringe holder cylindrical aluminium shapes; (2) syringe barrel; (3) plunger holding screw cap; (4) threaded end piece for 3; (5) syringe tightening screw; (6) EDP-2 stepper motor. C: the controller, the syringe and the modified EDP-2 micropipettor assembled.

mm, inner diameter 0.8 mm) was then used to make a tight seal onto the pipette, by compressing it between the Teflon washers in the end-cap of the syringe. Injection pipettes were drawn by a micropipette puller (Narishige, Model PN-3) at maximal magnet force with the coarse filament heater at position 8 using 10 μl borosilicate

microcapillary pipettes (Fisher-brand Cat. No. 21-164-2c). The pipettes were dry-heat sterilized for at least 1 h and the outer tip diameter was broken with a flamed scalpel blade to approximately 25–30 μm under a stereomicroscope at about a 60° angle perpendicular to the axis of the pipette.

Use of the injector

Assembly of the injector The screw cap, with the Teflon washers either side of the O-ring, was placed on the blunt end of the injection pipette before both the Hamilton syringe and the glass injection pipette were filled with water-saturated butanol. Saturated butanol was less viscous than paraffin oil used in previously published systems and facilitated the filling of the system without bubbles. The Hamilton syringe was then tilted vertically and its metal end-piece filled with saturated butanol by releasing the securing bolt (arrow, Fig. 2C) to allow free movement of the syringe barrel. The Hamilton syringe was then filled by slowly sucking the butanol solution into the syringe barrel. The injection pipette was backfilled with the butanol solution from a 22G needle attached to a tuberculin syringe. The completely filled injection pipette was then inserted into the butanol-filled metal end-piece of the Hamilton syringe and the O-ring and washers were pushed into place by tightening the screw cap. The injector was then returned to a horizontal position and held with two clamps to the magnetic base.

The oocyte holder unit One of the novelties of this instrument is the use of a movable cell-holder and a fixed injector unit. The holding pipette was mounted on a micromanipulator to allow the three-dimensional positioning of the oocyte, thus facilitating its proper alignment with the tip of the fixed injection pipette. Oocytes were held by gentle suction (about 3 inches of H_2O) in the opening of a Pasteur pipette, heated to form a rounded tip of about 0.6–0.8 mm internal diameter (Fig. 1B). The suction was adjusted by a regulating syphon so that the cell was firmly held without being damaged. Several dozen oocytes were picked up at a time in a dropper pipette from their storage vial and fed one by one into the opening of the cell holder for microinjection. With a little practice the oocytes could be positioned in the holding pipette so that their vegetal hemispheres faced the injection needle to minimize the possibility of injections damaging their nuclei. After injection, each oocyte was transferred to a second vial by pinching the suction tube to release the holding suction. For convenience, the holding pipette and its micromanipulator were mounted on a tilting base, so that the whole assembly could be swung forward through 45°, thus allowing the oocyte to be released into a collection vial placed immediately below.

Loading the injection pipette The mounting procedure described above ensured that the injection pipette was completely filled to the tip with butanol solution. The sample to be injected was stored in an Eppendorf tube which was fixed by plasticine to the arm of a micromanipulator to allow it to be brought up to the tip of the injection pipette. The shank of the pipette was then filled with mRNA, or other solution for injection, by withdrawing the syringe plunger, using the Inchworm or the pipettor to draw up the desired volume (usually 1–2 μl). The loading process was carried out under visual control to ensure a bubble-free interface between the

butanol and injection solutions. A disadvantage of the low cost system was that it could only withdraw a fixed volume: 1 or 2 μl, depending on whether a 5- or a 10-μl Hamilton syringe was attached to the stepper-motor. Also, the volume to be dispensed had to be preset prior to loading the injector, and could not be changed once the pipette was loaded.

Operating the injector The oocyte was impaled by the injection needle by using the micromanipulator to advance it towards the needle. Injection was then accomplished by advancing the Inchworm or the motorized pipette by a preset displacement to deliver the required volume, based on the calibration (see below) of the instrument. The injection speed was set on the Inchworm so that 50 nl was injected in about 1 s. Successful injection was confirmed by a visible swelling of the oocyte and by movement of the meniscus in the pipette. After waiting for about 5 s the cell was withdrawn from the needle and released into the collecting vial. After a little practice, 5–6 cells/min could be routinely injected.

Calibration of the injector

To determine the accuracy and reproducibility of the injector system, ^{125}I-labeled proteins (bovine serum albumin or Protein A, 8.2×10^4 cpm per μl) were loaded into the injection pipette as described above and different volumes injected into oocytes. Replicate measurements were obtained in six oocytes for each injection setting. After injection, oocytes were collected in tubes containing 1 ml of Ringer solution and counted to determine the amount of label ejected. These values were then used to calculate the ejected volume from the known activity of the original solution.

After a 1-h incubation at room temperature the Ringer solution was removed from the oocytes, and the oocytes and incubation solutions were separately recounted to determine the amount of label which remained within the oocyte, and that which leaked out. These experiments were performed using both intact ovarian follicles as well as collagenase-denuded oocytes (i.e., oocytes freed of their surrounding epithelial and follicular cell layers [11]).

Similar experiments were also performed to determine the accuracy of the system when label was ejected into 10-μl droplets of water.

Several groups of 20 oocytes were injected with rat cortex mRNA using this system as well as the "manual system" described by Coleman [7], and both daily survival and expression of the kainate receptor were determined.

Preparation of mRNA and electrophysiological recording

Poly-A$^+$ mRNA was extracted from rat brains as previously described [12]. Electrophysiological recordings were made using a standard two-electrode voltage clamp technique [12] five days after mRNA injection.

Oocytes were voltage clamped at -60 mV and currents were recorded in response to bath application of 10 μM kainic acid. Kainate evoked a smooth, non-desensitizing inward current in brain mRNA-injected oocytes, while non-injected oocytes showed almost no response [13]. Measurements of peak current were obtained from 3 to 5 oocytes, and were averaged for the various groups.

Results

Calibration of the injector Fig. 3 shows measurements of volumes of labeled protein solution ejected into 10 μl droplets of water using the Inchworm positioner. The ejected volumes varied linearly with the plunger displacement. The reproducibility, as measured by the standard deviation of the volumes ejected, was on average better than 8% in the 5 to 120 nl range. The system had somewhat less reproducibility between 5 and 50 nl (9.5%), but this improved to 6.4% over the 55 to 120 nl range.

Calibration of intra-oocyte injections Mean volumes of labeled protein solution injected into oocytes are shown in Fig. 4. The total injected volume again varied linearly with the plunger displacement, but the volumes delivered were somewhat less than those ejected into water droplets. The overall reproducibility was 6.3% over the 5 to 70 nl range, and was even more consistent for volumes greater than 20 nl (e.g., reproducibility of 4.7% at 23 nl, and 2.3% at 56 nl). These reproducibility data are superior to those obtained by previously published systems which were in the 4.7–20% range [7–11].

To determine how much of the ejected label was retained within the oocyte, the cells and bathing solution were separately counted after a 60-min incubation. Results are shown in Fig. 4. Leakage of the label over the 5 to 70 nl range was on average 5.6% $\pm$ 2.5% (SD) of the total volume ejected. However, injection above 60 nl caused greater leakage (at 56 nl, 4.9%; at 70.8 nl, 10.3%).

Oocytes are normally surrounded by several layers of cells and connective tissue but, with the exception of the vitelline envelope, all these can be removed by treatment with collagenase [12]. Injections into collagenase-treated oocytes were also performed and compared with the injections into untreated oocytes from the same

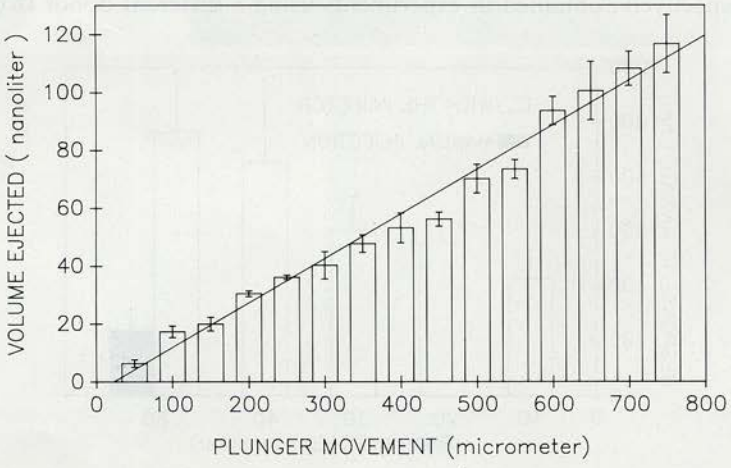

Fig. 3. Reproducibility of the volume delivery by the injection system. Various plunger movements were set by the Inchworm controller and labeled proteins were ejected into 10 μl water droplets. Bars represent the mean and standard deviation of 3 determinations.

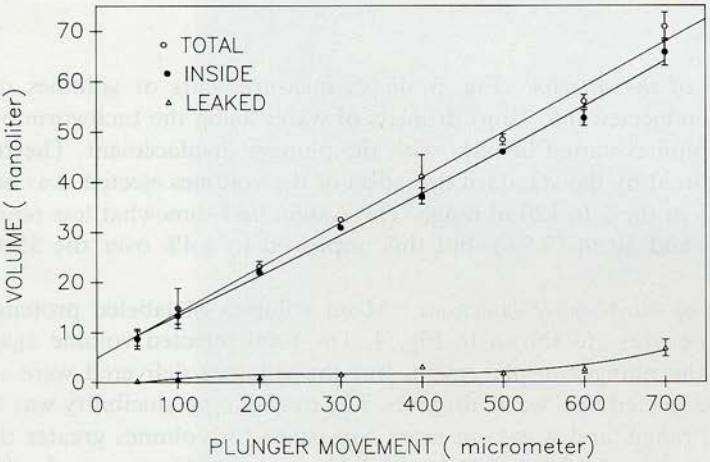

Fig. 4. Calibration of intra-oocyte injections. Open circles show the total volume of injected label, filled circles show the amount of label that remained inside the cell after a 1-h incubation in Barth's solution, and triangles represent volumes of label that leaked out during the first hour after injection. Each point shows mean and standard deviations from six oocytes.

donor. No significant differences in the reproducibility (e.g., 29.2 ± 0.2 nl versus 29.0 ± 1.2 nl, $n = 6$) or in the leakage (5.3 ± 2.0 nl versus 6.3 ± 0.5 nl at 55 nl) of the injected volumes were found between untreated and collagenase-denuded oocytes, respectively.

Expression of kainate receptors in rat brain mRNA-injected oocytes Oocytes injected with brain mRNA using the injection system described survived better than those injected using our previous manual system (see [7] for the instrument), more than 90% of oocytes ($n = 205$) surviving after 5 days, compared to 75% ($n = 50$) survival respectively, obtained in experiments using 3 different donor frogs.

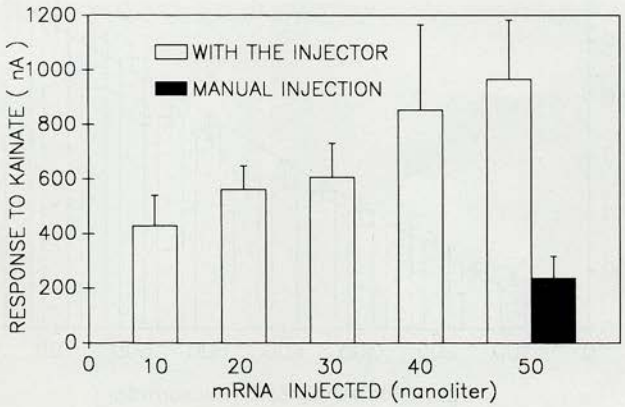

Fig. 5. Currents evoked by 10^{-4} M kainate in rat cortex mRNA-injected oocytes. Open bars show mean $\pm$ SD response sizes recorded from groups of 3–5 oocytes injected with the injector. Filled bar shows the mean response from cells ($n = 4$) injected manually with 50 nl mRNA.

The average sizes of induced responses to 10^{-4} M kainate in oocytes injected with increasing amounts of rat cortex mRNA are shown in Fig. 5. The response size increased linearly with the amount of mRNA injected. Oocytes that were manually injected with mRNA showed considerably smaller responses to kainate compared to oocytes that were injected with a comparable amount of mRNA using the present device (Fig. 5), possibly because the manual injection caused more damage to the oocyte and allowed a greater leakage of mRNA.

Discussion

Using readily available components a semi-automatic system was developed for intracellular injection of *Xenopus* oocytes. The two main novelties of the instruments are the three dimensionally movable suction-operated oocyte holder, and the programmable fixed injector. The few non-commercially available parts for the Hamilton syringe holder can easily be made in any laboratory machine-shop. The first version of the system used an Inchworm translator to drive the injection syringe, which allowed continuous adjustment of the injection's volume. However, this translator is expensive, and in a second version was replaced by the drive from an inexpensive motorized pipettor (EDP-2) without appreciably impairing the function or ease of use of the system. The injector requires little skill to operate, yet gives highly accurate volume delivery and reproducibility combined with superior survival of the injected oocytes. Oocytes can be injected as rapidly as six per minute, permitting the injection of large numbers of oocytes for biochemical or immunological experiments. Rapid and simple exchange of the injection pipettes allows the injection of multiple samples in a short period of time.

The results described above show that the system was capable of 6.3% average reproducibility over the 5 to 70 nl range. The volumes injected into oocytes for a given plunger movement were less than those injected into water droplets. There are no clear differences in injected volumes or in leakage between normal and collagenase-treated oocytes, but for routine work we prefer to make injections into untreated oocytes, as these are more robust, easier to handle and survive better longer periods of culture.

Measurement of the expression of kainate-activated channels in rat cortex mRNA-injected oocytes further supports the superiority of the present injector over our previous manual device. The lack of lateral movement of the holder during the injection process may be responsible, at least in part, for the enhanced survival and expression found in these cells. Manual systems, where the oocyte is held by forceps or sucked into a pipette for transfer following injection, are likely to impose more mechanical stress on the oocytes, e.g., often leading to visible leakage of yolk during the insertion and removal of the injection pipette.

From the calibration experiments using radioactive label we concluded that the optimal injections are obtained with volumes between about 20 and 50 nl. The reproducibility of volume delivery is maximal over this range, while leakage from the oocyte is minimized with an optimal cell survival.

Simplified description of the method and its application

Oocytes from the frog *Xenopus laevis* are now widely used as a translation system for the expression of membrane receptors and ion channels coded by mRNAs derived from tissues and cloned genes. In order to make quantitative comparisons, it is important that oocytes be reproducibly injected with a given amount of mRNA solution, and that the injection process causes as little damage as possible. We describe here a new instrument for making precise and reproducible injections into oocytes. The principal novelties of this system are that the injection volume is set by a digitally programmable translator, and the oocyte is held by a suction pipette to minimize damage. It offers several advantages over a widely used manual injection system, including greater reproducibility, improved ease and speed of use, and a better rate of survival of oocytes after injection. Furthermore, we demonstrate by the use of the injector that the optimal volume for injection of mRNA solutions into the oocyte is about 50 nl, because injections of greater volumes result in a disproportionately high leakage from the injection site.

Acknowledgement

The authors would like to thank Prof. Ricardo Miledi for his support and valuable suggestions in designing the equipment.

References

1 Gurdon, J.B., Lane, C.D., Woodland, R. and Marbaix, G. (1971) Use of frog eggs and oocytes for the study of mRNA and its translation products in living cells. Nature 233, 177–182.
2 Miledi, R. and Sumikawa, K. (1982) Synthesis of cat muscle acetylcholine receptors by *Xenopus* oocytes. Biomed. Res. 3, 390–399.
3 Miledi, R., Parker, I. and Sumikawa, K. (1988) Transplanting receptors from brains into oocytes. In: FIDIA Neurosci. Award Lecture Series, Vol. 3, pp. 30–45.
4 Lubbert, H., Hoffmann, B.J., Snutch, T.P., van Dyke, T., Levine, A.J., Hartig, P.R., Lester, H.A. and Davidson, N. (1987) cDNA cloning of a serotonin 5-HT1c receptor by electrophysiological assays of mRNA-injected *Xenopus* oocytes. Proc. Natl. Acad. Sci. USA 84, 4332–4336.
5 Julius, D., MacDermott, A.B., Axel, R. and Jessel, T.M. (1988) Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. Science 241, 558–564.
6 Dascal, N. (1987) The use of *Xenopus* oocytes for the study of ion channels. Crit. Rev. Biochem. 22, 317–387.
7 Coleman, A. (1984) Translation of eukaryotic messenger RNA in *Xenopus* oocytes. In: Hames, C. and Higgens, R. (Eds.), Transcription and Translation–A Practical Approach, IRL Press, Oxford, pp. 271–302.
8 Hitchcock, M.J.M. and Firedman, R.M. (1980) Microinjection of *Xenopus* oocytes: an automated device for volume control in the nanoliter range. Anal. Biochem. 109, 338–344.
9 Conteras, R., Cheroutre, H. and Fiers, W. (1981) A simple apparatus for injection of nanoliter quantities into *Xenopus laevis* oocytes. Anal. Biochem. 113, 185–187.
10 Stephens, D.L., Miller, T.J., Silver, L., Zipser, D. and Mertz, J.E. (1981) Easy-to-use equipment for the accurate microinjection of nanoliter volumes into the nuclei of amphibian oocytes. Anal. Biochem. 114, 299–309.
11 Hitchcock, J.M., Ginns, E.I. and Marcus-Sekura, C.J. (1987) Microinjection into *Xenopus* oocytes: equipment. Methods Enzymol. 152, 284–288.
12 Sumikawa, K., Parker, I. and Miledi, R. (1989) Expression of neurotransmitter receptors and voltage-activated channels from brain mRNA injected oocytes. In: Conn, P.M. (Ed.), Methods in Neurosciences, Academic Press, Florida, pp. 30–45.
13 Gundersen, C.B., Miledi, R. and Parker, I. (1984) Glutamate and kainate receptors induced by rat brain messenger RNA in *Xenopus* oocytes. Proc. R. Soc. London Ser. B 221, 127–143.