ELSEVIER

Journal of Neuroscience Methods 169 (2008) 65–75

JOURNAL OF NEUROSCIENCE METHODS

www.elsevier.com/locate/jneumeth

An automated system for intracellular and intranuclear injection

Ron C. Hogg [a,*], Florence Bandelier [a], Audrey Benoit [a], Roland Dosch [b], Daniel Bertrand [a]

[a] *Department of Neurosciences Fondamentales, Medical Faculty, University of Geneva, Switzerland*
[b] *Département de Zoologie et de Biologie animale, University of Geneva, Switzerland*

Received 20 July 2007; received in revised form 22 November 2007; accepted 23 November 2007

Abstract

The *Xenopus* oocyte expression system has played an important role in the study of cellular proteins, particularly in the field of membrane physiology; expression of transporters and ion channels has significantly advanced our knowledge of these membrane proteins and the rapid and easy expression of mutants has been crucial in many structure–function studies. *Xenopus* oocytes are an expression system in many ligand-binding assays and in functional screening for ion channel modulators. Several commercially available automated technologies use this system, generating a demand for large numbers of oocytes injected with ion channel genes. Injection of oocytes with genetic material is generally carried out manually. Here we describe an automated system capable of injecting up to 600 oocytes per hour. Oocytes are contained in microplates with conical wells, a simple calibration procedure by the operator is required and pipette filling and oocyte injection are carried out automatically. Following intracellular injection of mRNA coding for ligand-gated ion channels close to 100% of oocytes tested positive for expression, and intranuclear injection of cDNA gave a rate of expression >50%. Moreover, we demonstrate that this method can also be successfully applied to inject zebrafish embryos and could be extended to other cell types.

Keywords: *Xenopus* oocyte; Zebrafish; Intranuclear injection; RNA injection; Electrophysiology; Lab automation; Drug screening

1. Introduction

Investigating the function of a particular cellular protein such as a membrane transporter, ion channel, metabotropic receptor or kinase in a native cell is often difficult due to the large number of interfering factors, such as similar proteins, present in the native cell. The identification and cloning of genes coding for many of these proteins has been an important step that has enabled researchers to express the resulting proteins in host cell expression systems. One of the principal advantages of expressing cloned genes in a host cell system is that it enables the study of the resulting proteins in relative isolation. Thus, host cell systems are chosen not only for their ability to express the genes of interest, but also for their lack of endogenous proteins which could make studies of the expressed protein difficult.

Oocytes from the south-African clawed frog *Xenopus laevis* were first used as an expression system for cellular proteins in 1971, where it was demonstrated that they were able to synthesise haemoglobin following intracellular injection of the corresponding mRNA (Gurdon et al., 1971). Subsequently it was demonstrated that they were capable of expressing a number of different proteins such as globin, interferon and viral proteins (Gurdon et al., 1973, 1974; Laskey and Gurdon, 1974; Laskey et al., 1972; Woodland et al., 1974) and in 1982 it was reported that, following injection of mRNA coding for acetylcholine- and GABA-sensitive receptors, *X. laevis* oocytes expressed fully functional channels with properties similar to those in native cells (Miledi et al., 1982a,b, 1983). Although early studies involved the injection of mRNA, Wickens and colleagues demonstrated that intranuclear injection of cDNA in *X. laevis* oocytes also resulted in protein expression (Wickens et al., 1980). It was sufficient that the gene of interest was in the correct orientation between a eukaryotic promoter such as SV40 and the SV40 transcription termination sequence, and transcription, capping, polyadenylation and export of mRNA from the nucleus were carried out by the oocyte. Moreover, cDNAs containing introns were correctly spliced and expressed by the oocyte (Bertrand et al., 1991). Although the injection of cDNA into the nucleus of the oocyte requires more precision than cytoplasmic injection, this technique eliminates the need for

* Corresponding author at: Department of Neurosciences, CMU, 1 rue Michel Servet, CH-1211 Geneva 4, Switzerland. Tel.: +41 22 379 53 55; fax: +41 22 379 54 02.
E-mail address: Ronald.Hogg@medecine.unige.ch (R.C. Hogg).

0165-0270/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneumeth.2007.11.028

in vitro transcription of cRNA. An additional advantage is that cDNA is highly stable and does not require the careful storage and handling necessary for cRNA.

As an expression system for cellular proteins, *Xenopus* oocytes have a number of advantages over mammalian cell lines. The frogs cost relatively little and are easily maintained and reproduce in captivity. Oocytes can be harvested many times from the same frog by partial ovarectomy and can survive outside the animal for up to a month if conserved at 4 °C and due to their large size; handling of the oocytes is easy. *Xenopus* oocytes have the necessary enzymes for the expression of a wide range of mammalian proteins and the oocytes are compatible with many bioassays, making them particularly useful in scanning mutagenesis studies that require the expression and functional testing of large series' of mutant proteins, as well as the pharmacogenomic application of screening existing drugs or drug candidates across proteins resulting from genetic variations, such as single nucleotide polymorphisms. Despite its numerous advantages, the *Xenopus* oocyte expression system also has several disadvantages. The most important of these is whether ion channels expressed in amphibian cells are assembled and behave in an identical fashion to those expressed in mammalian cells. Single channel experiments comparing the properties of rat α3β4 nAChRs expressed in oocytes with a stably expressing mammalian cell line (L-α3β4) reported that α3β4 receptors expressed in the mammalian cell line more closely resembled native channels from rat superior cervical ganglion than those in oocytes (Lewis et al., 1997; Sivilotti et al., 1997). It is not clear if these differences are due to a different stoichiometry of the channel subunits in oocytes or due to different posttranslational modification or functional modulation, such as phosphorylation, by the amphibian cell machinery. Additional challenges are posed by their large size, which gives a relatively slow fluid exchange time around the oocyte compared to a mammalian cell, this can be a problem for recording fast-desensitizing ligand-gated channels in oocytes. During long incubations with lipophilic compounds these can accumulate in the oocyte and slowly leak out after the compound has been removed preventing washout. Another disadvantage is that presently, each oocyte must be individually injected with genetic material, which is slower than the simultaneous transfection of large numbers of mammalian cells.

One area of research where the *Xenopus* oocyte expression system is widely used is in the study of ion channels. A main advantage is that most cDNAs and cRNAs are readily expressed without the need to develop a cell line, whereas there is often a significant delay from the cloning of a new receptor until it can be expressed in a mammalian cell line. Studying the function of an ion channel in a native cell often requires substitution of the ions present in the physiological recording solutions with impermeant ions to suppress endogenous currents which can mask the current of interest. This can result in significant effects on the properties of the ion channel being studied and is often not sufficient to remove the interference of other channels that are permeable to the same ions. In some cases interferance from endogenous channels and receptors can be minimised by having selective blockers of these ion channels in the recording solution; however, this is dependent on the availability of such compounds and for many ion channels selective blockers are not available. *Xenopus* oocytes contain few endogenous ion channels and transporters that can interfere with measurements of the protein of interest. Following injection of genetic material into the oocyte, proteins are expressed after 1–2 days and functional studies of ion channels and receptors are easily performed using conventional two-electrode voltage clamp techniques. The only consideration, is that due to the large size of the oocyte, the amplifier must be able to deliver sufficient current to clamp the transmembrane voltage in the voltage clamp configuration, which is now achieved with commercially available amplifiers.

The *X. laevis* expression system is used routinely for screening applications in drug discovery using expressed ion channel targets. Presently, there are several fully and semi-automated oocyte electrophysiology recording systems available commercially: Roboocyte (http://www.multichannelsystems.com) is fully automated and records from oocytes in a 96-well microplate; OpusXpress 6000A (http://www.moleculardevices.com) can record up to eight oocytes in parallel and is designed primarily for drug screening applications; and ScreenTool (http://www.npielectronic.com) is also designed for screening of compounds and combines electrophysiological recording and automated liquid handling. These machines require the injection of large numbers of oocytes with genetic material coding for target proteins, which can be an arduous task if carried out manually. The principal aim of this work was to design an injection system to automate this procedure in *Xenopus* oocytes and which could be extended to other cell types, such as the injection of genetic material or sperm in embryos from Zebrafish, *Drosophila* and ultimately mammals. Embryos of zebrafish and *Drosophila* contain a large number of identical genes and processes to those found in higher mammals making them widely used in studies of development and in disease. In addition genetically altered zebrafish are now used in a number of toxicological and drug screening assays, generating a need for new genetic mutants. The challenge of extending this method to other cell types will be in part dictated by the cell size, zebrafish embryos have a diameter of around 1.2 mm, which is similar to that of *Xenopus* oocytes, *Drosophila* embryos are 500 μm in length, whereas a human egg has a diameter of 100 μm. Thus we will commence by the largest cell types.

This work describes the design and validation of a fully automated system suitable for intracellular and intranuclear injection of cDNA and cRNA in *Xenopus* oocytes and zebrafish embryos.

2. Methods

2.1. Preparation of Xenopus oocytes

X. laevis were sacrificed according to Swiss National guidelines. Ovaries were removed and placed in Barth's medium, which contained 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 1 mM Hepes, 0.8 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, kanamycin 20 units/ml and penicillin-streptomycin 100 units/ml adjusted to pH 7.4 with NaOH. Ovaries were kept

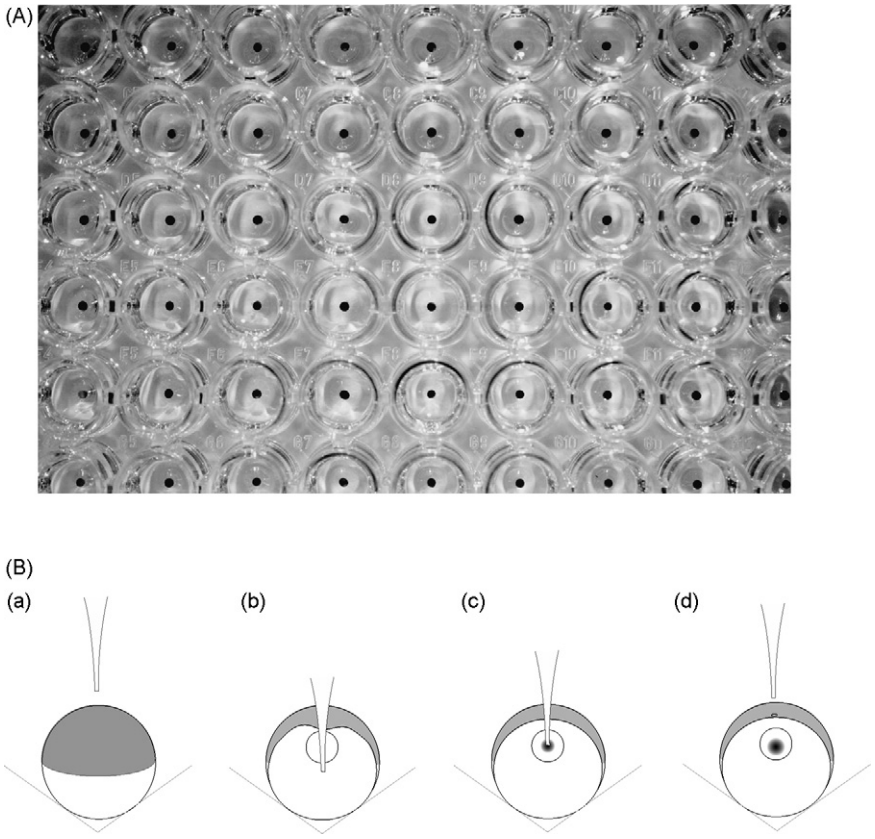

Fig. 1. Oocytes in a 96-well plate and schematic illustration of the injection protocol. (A) Oocytes pipetted into conical wells fall with the dark animal pole uppermost and are centered in the well. (B) a: the injection pipette is positioned over the centre of the conical well containing the oocyte; b: the pipette is rapidly lowered to 0.5 mm above the bottom of the well, which corresponds to the approximate midpoint of the oocyte, this ensures that the elastic membrane is penetrated; c: the pipette is raised until the tip is 0.8 mm above the bottom of the well, which corresponds to the position of the nucleus and the cDNA is ejected from the pipette; d: the injection pipette is withdrawn from the oocyte.

at 4 °C and the bathing solution was changed weekly. Under these conditions oocytes remained viable for up to 4 weeks. Prior to injection, oocytes were defolliculated by incubating in Ca^{2+}-free OR2 solution (82.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1 mM MgCl, adjusted to pH 7.4 with NaOH) containing 0.2% collagenase, type 1 (Sigma–Aldrich) with slow agitation for 3–4 h. Following defolliculation, oocytes were washed with standard OR2 solution (82.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 2.5 mM $CaCl_2$, 1 mM MgCl, pH 7.4) solution to remove any remaining collagenase activity and kept in Barth's medium. Generally stage V or VI oocytes were manually selected for injection under a binocular microscope, and deposited in the wells of a 96 or 384 conical well microplate which was pre-filled with Barth's medium. Several oocytes were aspirated in a Pasteur pipette and one oocyte was deposited in each well by touching the tip of the pipette on the liquid surface.

2.2. Preparation of zebrafish embryos

Zebrafish were kept and raised as described (Brand et al., 2002; Westerfield, 2000). To obtain uniformly staged embryos, a pair of *AB or TÜ wild type fish were placed together in a mating box, but separated by a barrier. For spawning the next day, the pair was combined, embryos were collected in 1×E3 (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ and 10^{-5}% methylene blue) and staged as described (Kimmel et al., 1995).

2.3. Requirements and design of the machine

Our objective was to develop a fully automated system for the intracellular and intranuclear injection of cDNA and cRNA. Our requirements were that the system could be operated by untrained personnel, with a minimum possibility of operator error, and that in the case of *Xenopus* oocytes the number injected per hour should be greater than the 300–400 possible with a manual injection by a skilled person. We have considerable experience with manual injection in *Xenopus* oocytes. Intranuclear injection of cDNA requires the greatest precision from the operator, the injection point, in the centre of the animal pole, is identified by eye and the position of the oocyte, angle and depth of injection are all controlled by the technician. All of these parameters are crucial for correct positioning of the tip of the injection pipette in the nucleus and require experience. To automate this process, a positioning system with at least three axes and sufficient resolution and repeatability to accurately position the tip of the pipette in the nucleus was required. To insert the injection pipette in the oocyte at the correct point, we considered two options, the first was to capture an image of the oocyte

and identify the injection point using an image recognition system and to calculate its coordinates and direct the pipette tip to this point using a multi-axis positioner. This approach requires an image capturing system and algorithms for the identification of the injection point and calculation of coordinates from the captured image. The second option was to locate the oocyte precisely by placing it in a depression, thus, the coordinates for each oocyte are determined by the location of the depression. As a suitable guide to position oocytes we tested commercially available microplates with conical wells. The spacing between the wells of the microplate determines the displacements of the injection pipette in the *X*- and *Y*-axes. This approach does not require an imaging system and does not require the calculation of coordinates as these are obtained from the microplate specifications. If we are to avoid changing the angle of the injection pipette the orientation of the oocytes must all be identical. As the vegetal pole of the oocyte is heavier than the animal pole oocytes fall with the animal pole uppermost when dropped into a well filled with fluid, and are centred by the conical well (see Fig. 1A and B). The oocyte nucleus has a diameter of approximately 0.2 mm and is located directly under the centre of the animal pole a depth that corresponds to approximately 30% of the diameter of the oocyte. We determined the variation between the centre of the oocyte animal pole and the centre of the well by placing a microplate containing oocytes on a high precision micrometer driven *X*–*Y* table and locating the centre of the oocyte using an eyepiece with concentric graticule. The difference between the centre of the oocyte and the centre of the well was measured in the *X* and *Y* planes. More than 80% of oocytes dropped into a 96-well microplate were located with the centre of the animal pole less than 0.1 mm from the centre of the well in the *X* and *Y* planes. Thus, if the injection pipette is lowered vertically in the centre of the well it should enter the nucleus of at least 80% of oocytes. Oocytes that were found not to lie centrally in the conical well were usually misshapen and this could be minimised by visual inspection and rejection of non-spherical oocytes during the selection stage. A total of three axes are required to inject by lowering the injection pipette vertically into the oocyte, therefore, we chose this second option to keep the complexity of the machine to a minimum.

2.4. Description of the automated machine

We constructed an injector based around an *X*–*Y* positioner (Multichannel systems, MCS GmbH, Reutlingen, Germany) (Fig. 2). The microplate carrier (Fig. 2(a) and Fig. 3A) moves on an air cushion controlled by linear stepper motors with a step resolution of 1 μm. A *Z*-axis was added to move the injection pipette vertically in relation to the microplate and is moved by a linear actuator (L4218M1404-T6x2 Nanotec, Nanotec Electronic GmbH & Co. Landsham, Germany) controlled by a 30X microstepping driver (Multichannel systems, MCS GmbH, Reutlingen, Germany) to give a final resolution of 0.33 μm per microstep. Filling and emptying of the injection pipette is carried out by a microdispenser (3-000-105, Drummond Scientific, Broomall, PA, USA) with a maximum ejection volume of 5 μl. A precision stainless steel wire plunger moves in a calibrated glass capillary tube forming an airtight fit. Plunger travel is controlled by a linear actuator (Z26000, Haydon Motion Europe, Coueron, France) with a step resolution of 1 μm controlled by a microstepping driver (Multichannel systems).

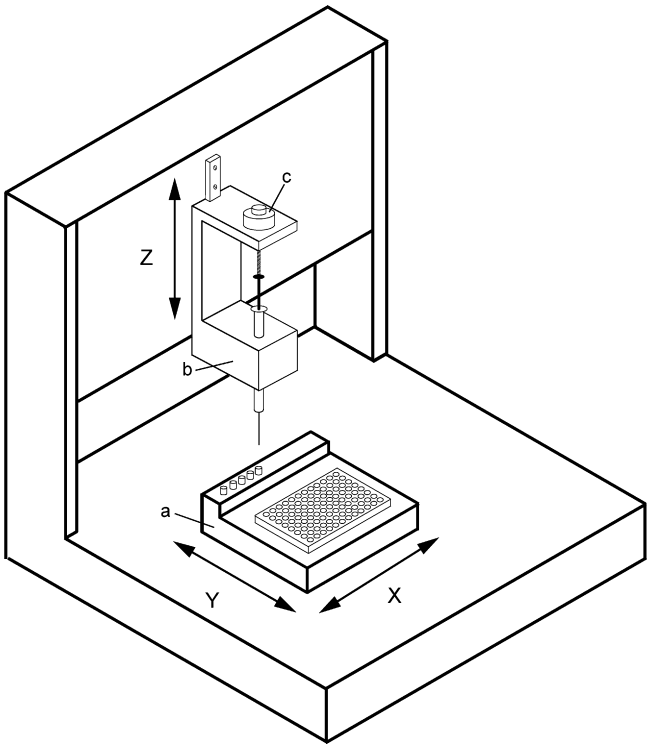

Fig. 2. Schematic illustration showing the main parts of the injection machine. The tube rack and microplate containing the cells to be injected are located on the microplate carrier, which floats on an air cushion and moves in the *X* and *Y* planes. The assembly (b) holds the microdispenser syringe and moves in the *Z* plane on a precision rail controlled by a linear actuator. A second linear actuator (c) controls the movement of the microdispenser piston.

2.5. Functioning of the machine

2.5.1. Set up procedure

Injection pipettes were prepared from calibrated borosilicate capillary glass (3-000-105G, Drummond Scientific, Broomall, PA, USA) and pulled to a fine point on a horizontal puller (BB-CH-PC, Mecanex, Switzerland). Pipette tips were broken with forceps to give a tip that was as fine as possible without being prone to blocking (outside diameter of approximately 40 μM). The pipette was backfilled with mineral oil (Sigma–Aldrich) and slid onto the plunger wire ensuring that no air bubbles were present between the oil and the plunger. When the pipette was pushed fully home a retaining collet was tightened to prevent any movement of the pipette when the plunger is moved.

2.5.2. Calibration for the microplate

With the injection pipette in place the operator performs a single calibration procedure, positioning the pipette tip at a reference position on the microplate. An aluminium guide with a central marking (Fig. 3A) is inserted in a corner well, the

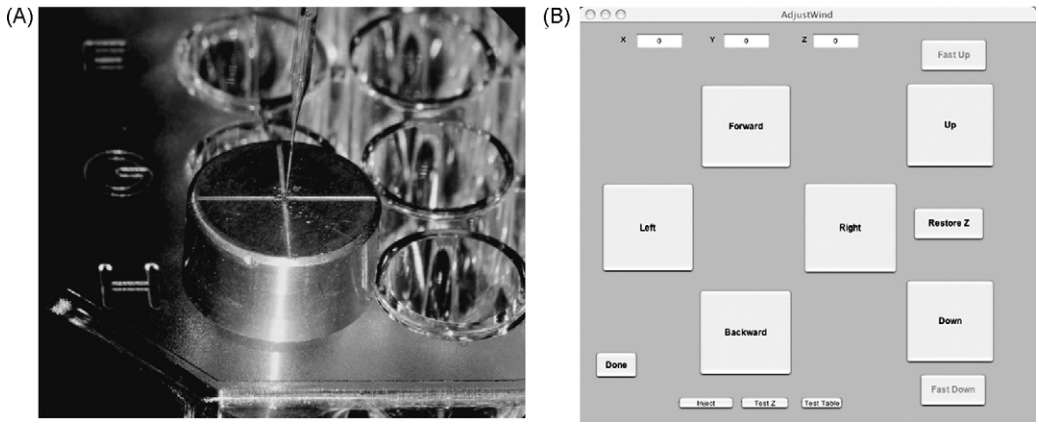

Fig. 3. Photograph of calibration guide and on-screen control pad for adjusting the reference position of injection pipette. The injection pipette is adjusted to the centre of the cross using the on-screen control pad.

pipette tip is positioned on the central marking with the on-screen controls (Fig. 3B) and the reference point is stored by the program. This procedure is carried out under 100–125× magnification using a stereo-microscope. Since all positions, including the injection point, tubes containing genetic material and the wash position are fixed relative to this reference point no further calibration or intervention by the operator is necessary.

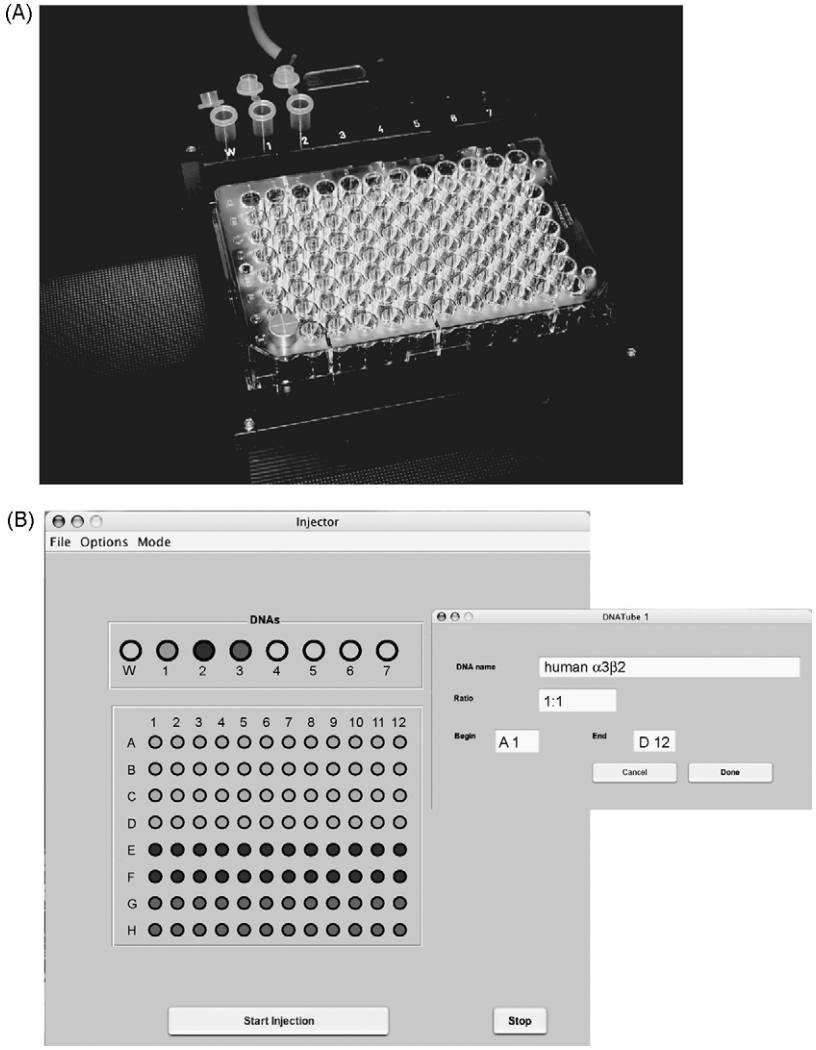

Fig. 4. (A) Illustration of the microplate carrier showing a 96-well microplate with the calibration guide and tube rack for the rinse solution and cDNAs. (B) Screen-capture of the window for entry of the cDNA information and selection of the wells to be injected.

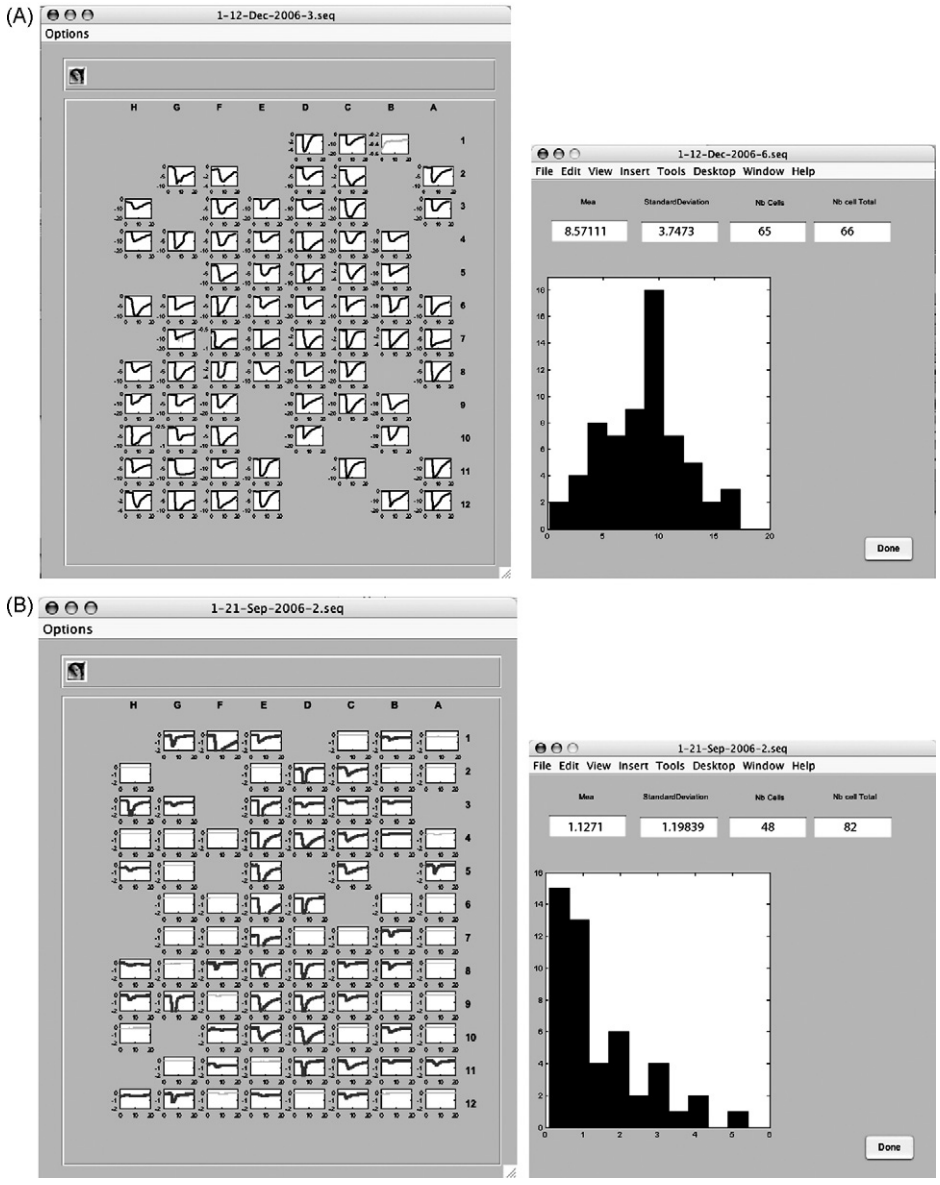

Fig. 5. Test of microplates injected with genetic material. (A) Test of a 96-well microplate injected with mRNA coding for the *Torpedo* nAChR. Oocytes were challenged with 30 μM acetylcholine and displayed a current if they expressed the receptor. Oocytes that responded are indicated by a dark line, whereas oocytes that were not successfully tested are indicated by blank spaces, 65 of 66 oocytes responded, mean current amplitude was 8.5 ± 3.7 μA. The window to the right shows the distribution of the number of oocytes displayed as a function of current amplitude in μA. (B) Test of a microplate injected with cDNA coding for the human α4β2 nicotinic acetylcholine receptor. The mean current amplitude was 1.4 ± 1.1 μA and 48 of 82, corresponding to 59% of oocytes responded.

2.5.3. Protocol selection

Eppendorf tubes (0.5 ml) containing the pipette rinsing solution (water) and the genetic material for injection are inserted in the tube rack (Fig. 2 and Fig. 4A). The user indicates the location of the tube containing the material to be injected by clicking on the display and identifies it with a name (Fig. 4B). The rows and columns to be injected are selected by entering the position of the first and last wells in the series. Several cDNAs can be selected sequentially for injection in different areas of the same microplate. Pressing the start injection command automatically directs the injection pipette to the correct tube and aspirates the solution to be injected. The volume of solution aspirated is calculated based on the number of oocytes selected for injection by the user. The machine then carries out a sequential injection of the selected oocytes (see Fig. 1). When multiple cDNAs are to be injected in the same microplate, the injection pipette is automatically rinsed with water following the first series of injections and the next cDNA is aspirated before resuming the injection for the defined series of oocytes. The maximum number of cDNAs that can be injected in the same microplate is determined by the number of places in the tube rack, which is presently seven. The calibration and protocol selection procedure are similar for 96- and 384-well microplates. When the 384-well mode is selected, the 384-wells are displayed on the screen for selection. A guide designed to fit the well of the 384-well microplate is used for calibration.

2.5.4. Injection protocol in detail

The average diameter of a stage V–VI oocyte is around 1.2 mm, after defolliculation the oocyte remains surrounded by a fibrous vitelline membrane, which is slightly elastic. When the oocyte is positioned, with the animal pole uppermost, the centre of the nucleus is located approximately 0.8 mm above the base of the microplate. To ensure that the injection pipette penetrates the slightly elastic membrane of the oocyte the tip of the pipette is lowered at a speed of 60 mm/s to a point 0.5 mm above the base of the microplate, which corresponds approximately to the mid-point of the oocyte (Fig. 1B(b)). The pipette is then raised by 0.3 mm so that the tip of the pipette is in the nucleus and following a brief pause to allow for the relaxation of the membrane the desired volume of genetic material is expelled from the pipette (Fig. 1B(c)). The injection pipette is then raised and the microplate is moved in the *X* and/or *Y* planes to position the next oocyte under the injection pipette.

3. Results

3.1. cRNA injection

As a first validation of the injector we started with the injection protocol having the least stringent injection conditions. Many researchers using the *X. laevis* expression system inject cRNA coding for a protein of interest. Although cRNA requires more careful storage and handling than cDNA, it can be injected anywhere in the cytoplasm of the oocyte and thus requires less precision than for intranuclear injection of cDNA. Moreover, cRNA injection generally gives a higher percentage of oocytes that express the gene compared to cDNA, and the time from injection to protein expression is shorter. Using the injection parameters described above we carried out an automatic injection of cRNA coding for the nAChR from *Torpedo californica* in *Xenopus* oocytes. Two 96-well microplates were injected and the percentage of oocytes which displayed currents was 95% and 99%, respectively (Fig. 5A), clearly demonstrating that an automated injection is effective for the injection of cRNA in *Xenopus* oocytes.

3.2. Dye injection in Xenopus oocytes

The intranuclear injection of cDNA requires a higher degree of precision regarding the poisoning of the pipette relative to the centre of the oocyte. To find the optimal position of the tip of the injection pipette for intranuclear injection we injected coloured dye into oocytes at different depths. When the tip of the injection pipette was adjusted to a distance of 0.8 mm from the bottom of the microplate the dye was injected in the nucleus of the oocyte, see Fig. 6. This procedure to find the optimal depth for intranuclear injection was carried out for both 96- and 384-well microplates.

3.3. Intranuclear injection of cDNA

Based on previous experience in our laboratory a skilled and experienced person could typically expect a rate of expression between 40% and 80% from a manual intranuclear injection of cDNA into *Xenopus* oocytes. To test the rate of expression following automatic injection, oocytes were injected with cDNA coding for either human α4β2, α3β2 or α7 nAChRs. Oocytes were injected 2 ng of cDNA per oocyte in 10 nl of water, and 48–72 h after injection, oocytes were voltage clamped and challenged with 30 or 100 μM acetylcholine. The average number of oocytes responding per 96-well microplate was 51 ± 2% ($n = 13$) with a maximum of 67%. Of these thirteen, four plates were injected with cDNA coding for α4β2, three with α7 and six with α3β2. A typical microplate injected with α4β2 cDNA is shown in Fig. 5B. Several factors contribute to the success rate of an injection, the length of time since the ovary was harvested from the frog, the shape and health of the oocytes vary and some cDNAs express more readily than others. The injection parameters, such as the position of the injection point and speed of *Z*-axis movement also play an important role and were frequently adjusted during the initial tests to find the most favourable combination. Currently the maximum displacement of the plunger permits the injection of up to 400 oocytes (with a volume of 10 nl) before refilling of the injection pipette, which is carried out automatically. We have also used this automated method successfully for the injection of cDNA coding for voltage-gated ion channels with comparable results. Fig. 7 shows a microplate injected with

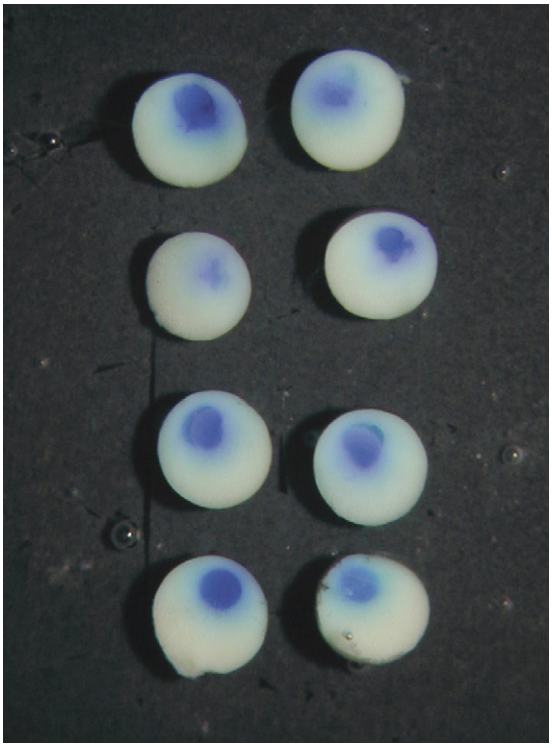

Fig. 6. Illustration of oocytes injected with coloured dye in the nucleus. Four oocytes (A–D) are shown following injection with coloured dye with the tip of the injection pipette adjusted to a depth of 0.8 mm above the bottom of the well. Following the injection, oocytes were placed in boiling water and then cut open. The two halves of the same oocyte are shown on the left and right hand sides of the image. On the right hand side the limits of the nucleus are indicated by the dotted line. The blue dye can be seen in the nucleus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

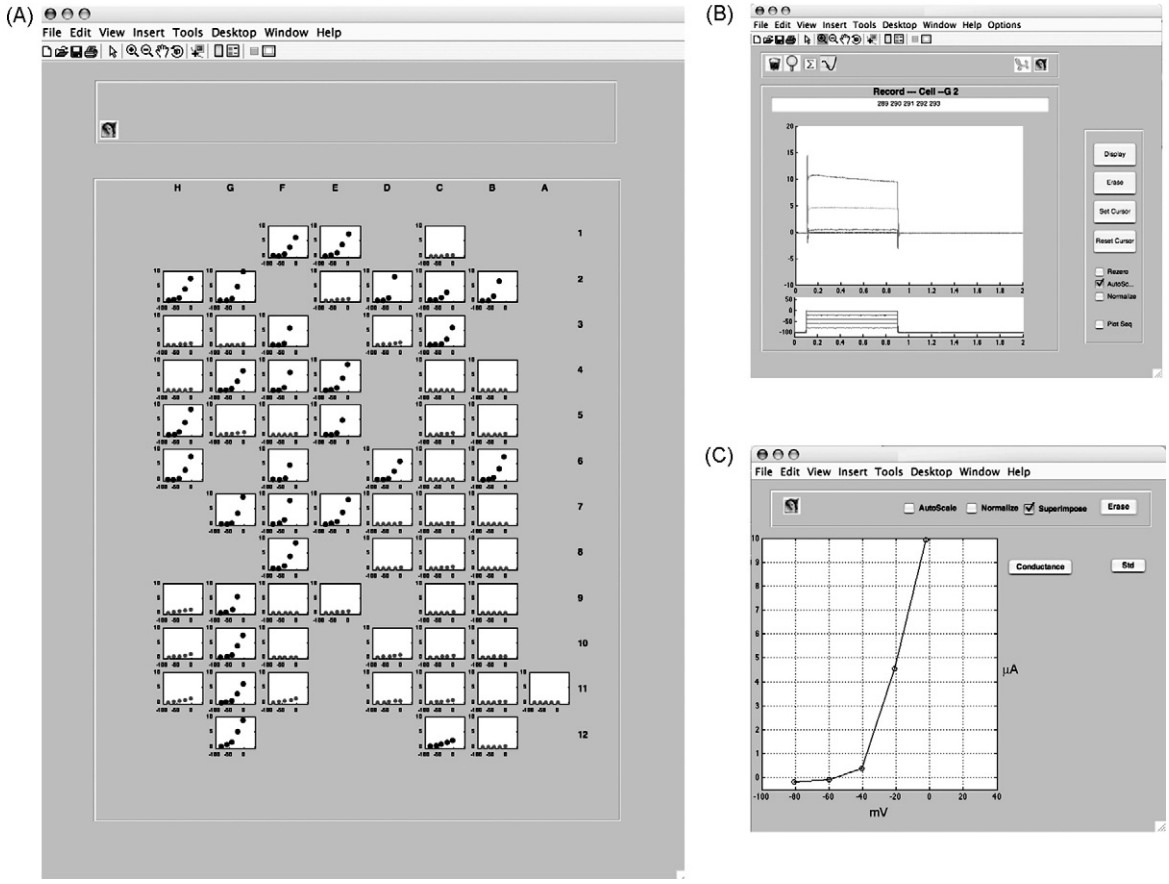

Fig. 7. Test of a microplate injected with cDNA coding for the voltage-activated potassium channel Kv1.7. Oocytes were voltage clamped and the membrane potential was held at −100 mV. Currents were activated by 1 s voltage steps from −80 to 0 mV in 20 mV increments. (A) Shows the current–voltage relationship for each oocyte; (B) shows the voltage command protocol and membrane currents recorded from the oocyte in the well G2; (C) shows the current–voltage relationship for the oocytes in well G2.

cDNA coding for Kv1.7 potassium channels, 43% of oocytes expressed a current (27/63). The current–voltage relationship for each oocyte is represented in Fig. 7A, the voltage-activated currents from the oocyte at position G2 are shown in Fig. 7B and the current–voltage relationship for this oocyte is plotted in Fig. 7B.

3.4. Injection of zebrafish eggs

To extend the possible applications of the automated injector we investigated the suitability for injection of fertilized embryos. The zebrafish, *Danio rerio*, is a widely used organism in the field of developmental biology, embryos are transparent and develop outside the fish allowing easy visualisation of organ development. Zebrafish have a short reproductive cycle and produce a large number of progeny, which can be easily maintained in a small space. The embryos develop rapidly, however, cells remain connected up until the 8-cell stage and genetic material injected in the yolk can enter cells by cytoplasmic streaming. A single embryo was placed in each conical well of a 96-well microplate, to visualise the injection point, we first injected test embryos with coloured dye, visual examination under a binocular microscope confirmed that the dye was injected in the yolk when the tip was centered and lowered to 0.5 mm from the bottom of the well. Fertilized embryos were injected with 3 nl of mRNA coding for green fluorescent protein (GFP) into the yolk. Twenty-four hours following injection, embryos were visually inspected using a binocular microscope equipped for fluorescence imaging. In two microplates 88% and 70% of the embryos were fluorescent (Fig. 8), confirming the feasibility of automated injection of genetic material in zebrafish embryos.

4. Discussion

In this work we demonstrate the feasibility of automated intranuclear and intracellular injection of *Xenopus* oocytes and zebrafish embryos. There are several points to consider for an automatic *versus* manual injection, such as the number of oocytes to be injected, the number oocytes per hour that can be injected with the machine *versus* manually, the success rate of the injection and the relative cost of the equipment. Techniques such as expression cloning and in-cell NMR spectroscopy and automated electrophysiological recording systems like OpusXpress require the injection of a large number of oocytes. Apart from requiring a skilled person to perform the injection, the task of injecting large series of oocytes is repetitive and tedious. In its present configuration, the machine is capable of injecting around 600 oocytes per hour, which is considerably quicker

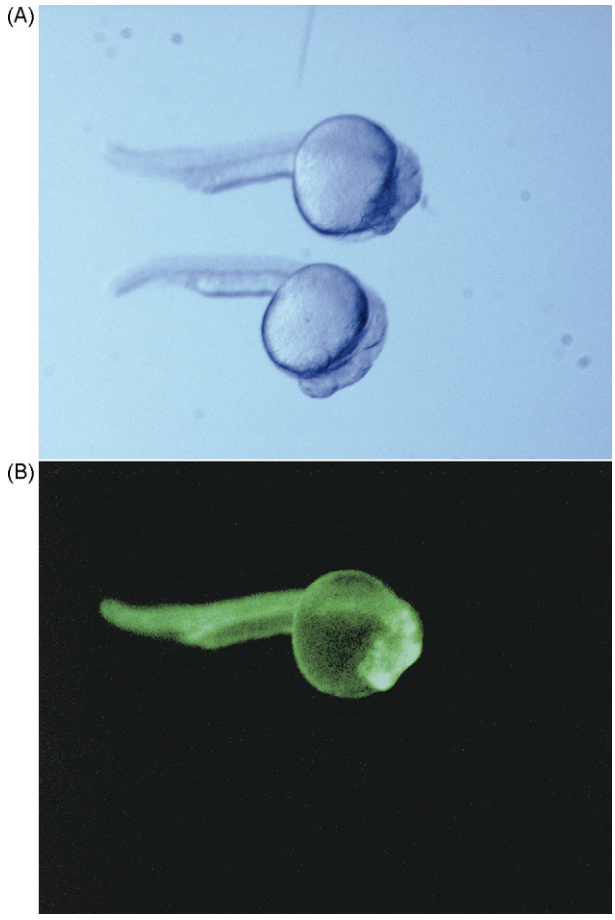

Fig. 8. Automated injection of zebrafish embryos. (A) Top, embryo injected with mRNA coding for GFP, and bottom, control uninjected embryo under brightfield illumination. (B) Same embryos photographed with UV illumination. Embryos were dechlorinated and photographed 34 h following injection.

and produces less operator fatigue than manual injection. However, the number of oocytes injected per hour could be increased by changing the injection parameters and motor commands. The simplicity and rapidity of the set-up and calibration procedure makes the automatic injection time effective even for the injection of a small number of oocytes, such as one 96-well microplate.

One important advantage of the automated injection is the reduced manipulation and potential for damage to the oocyte. In the manual injection procedure the oocyte is manipulated and held in position during the injection with fine forceps. Manipulation of the oocyte increases the possibility that the oocyte is damaged or contaminated. If the injection pipette is moved only forwards and backwards in one plane, considerable manipulation of the oocyte is necessary to place it in the required position for intranuclear injection. Alternatively if the injection pipette is controlled using a pantograph system there is the possibility that the pipette is moved laterally whilst in the oocyte, causing tearing of the oocyte membrane around the injection hole, leading to increased oocyte mortality. In the automated system the oocyte is immobilised in the conical well and the pipette is lowered and raised with no lateral movement, minimising damage to the oocyte. We observed better resealing of the injection point and improved oocyte survival following automatic as compared to manual injection.

Presently, the only commercially available system capable of automated injection of *Xenopus* oocytes is the roboocyte system. We believe that the present system brings new developments and addresses several shortcomings of the roboocyte. The roboocyte is not a dedicated apparatus for cell injection, it is a sophisticated system for automated electrophysiological recording from oocytes and comprises, a two-electrode voltage clamp amplifier, a perfusion system and dedicated data acquisition and analysis software, it is therefore not a cost effective solution for cell injection. The roboocyte, and a number of commercially available manual injection systems use air pressure to expel the contents of the injection pipette. We found that this method had several disadvantages compared with the plunger-driven injector used here, each injection pipette has a slightly different sized tip opening and therefore the pressure required to deliver a given volume must be calibrated by adjusting the air pressure and pulse duration to move the meniscus of the pipette solution by a known distance. This calibration must be repeated for each injection pipette. During a series of injections the pipette frequently becomes partially or fully blocked and due to the compressibility of air, the pressure may not be sufficient to expel the contents of the injection pipette. Thus, constant visual monitoring of the meniscus of the injection solution in the pipette is required to ensure that correct volume of solution is expelled during the injection pulse. For small injection volumes (3 nl), even under high magnification, visual monitoring is not a reliable control. Using the piston/oil system there is no compressible material between the plunger and the injection solution, preventing problems associated with blockage of the injection pipette. A further advantage of the piston driven system is that it allows automated filling and cleaning of the injection pipette, and up to seven different DNAs or RNAs can be injected on the same microplate without user intervention. Using the present system it is also possible to inject both 96- and 384-well microplates and we provide the first demonstration of successful automated injection in zebrafish eggs. Although the initial cost of an automated system as described here may be higher than that of a comparable manual system, the ease of use by untrained personnel and the number of operator hours saved make it a viable alterative to manual injection.

5. Applications

For the present validation of the automatic injector we have concentrated mainly on the injection of cDNA and cRNA coding for ligand-gated ion channels; however, injection of cRNA and cDNA coding for a variety of membrane and intracellular proteins, such as voltage-gated ion channels, metabotropic receptors, 7TM receptors, steroid receptors, transporters, aquaporins, and whole RNA injection is possible. Moreover, the simultaneous injection of several cDNAs can be used to investigate the effects of one recombinant protein on another.

A method of incorporating membrane proteins embedded in native lipid membranes into oocytes was also reported. Membrane proteins are “microtransplanted” into *X. laevis* oocytes

with their native cell membranes; the foreign membrane with functional receptors or ion channel is incorporated into the oocyte membrane. The effectiveness of this technique was first demonstrated using acetylcholine receptors and chloride channels from *Torpedo* electric organ (Marsal et al., 1995; Morales et al., 1995) and was extended to AMPA, GluR1, α7 and α4β2 acetylcholine receptors (Miledi et al., 2002, 2006; Palma et al., 2003). Membrane vesicles from native cells such as human hippocampus, temporal neocortex or from mammalian cell lines expressing receptors or ion channels of interest can be prepared and injected into the oocyte, extending the use of *Xenopus* oocytes as a system to study native membrane proteins.

The *Xenopus* oocyte expression system is ideally suited to gene isolation by expression cloning. Using this technique cDNAs are directly selected for their ability to produce proteins with a desired function or characteristics. The bioassay is selected according to the gene that is being researched and can be electrophysiological measurements, ion flux studies or labelling with antibodies or labelled probes. The method involves the isolation of poly(A)$^+$ RNA from a tissue which is injected into *Xenopus* oocytes and assayed with a functional test. The poly(A)$^+$ RNA is size-selected and the fraction giving rise to functional responses is used to produce a cDNA library which is in turn size-selected. This cDNA is amplified and used to prepare sense cRNA, which is re-injected into oocytes and functionally tested until a single clone is identified. A variety of genes for ion channels, transporters and enzymes have been isolated using this technique (see Romero et al., 1998). This technique requires the injection of many oocytes with cRNAs and automation of this step would significantly speed up the process.

Recently, *Xenopus* oocytes have been used for eukaryotic in-cell NMR spectroscopy (Sakai et al., 2006; Selenko et al., 2006). This technique exploits the fact most atomic nuclei in natural cellular molecules are inactive to NMR allowing isolation of NMR-active labelled protein samples. Labelled proteins were introduced into oocytes by microinjection and around 200 injected oocytes were required per sample. Moreover, NMR measurements have also been carried out in extracts from *Xenopus* oocytes, allowing higher quality measurements and greater control of the molecular environment see (Selenko and Wagner, 2007). This technique permits the study of protein structure in a cellular environment and enables researchers to follow structural changes and posttranslational modifications during cellular processes such as cell cycle progression, differentiation and apoptosis.

Gamarnik and Andino (1996) described a novel system to study virus replication in *Xenopus* oocytes. Poliovirus RNA microinjected into *Xenopus* oocyte underwent a complete cycle of viral replication, giving a high level of infectious viruses. Co-injection of HeLa cell extract was required to support replication and these factors could be identified (Gamarnik and Andino, 1996). This technique was later extended to other picornaviruses including human rhinovirus 14 and mengovirus (Gamarnik et al., 2000). Formation of infectious rhinovirus particles requires co-injection of human cell extracts. This technique is ideally suited to identify host factors which are necessary for viral replication and for biochemically dissecting the steps involved and is discussed in a recent review (see Gamarnik and Andino, 2006).

Zebrafish are widely used in assays of toxicity and teratogenicity; however, they are also an excellent system for molecular genetic analyses and forward-genetic approaches have resulted in a large number of monogenic human genetic disease models, and models of acquired diseases such as carcinogenesis, infection, immunological disorders, inflammation, wound healing, metabolic, endocrine, psychological and behavioural abnormalities, making them promising tools for drug discovery (Lieschke and Currie, 2007). Zebrafish can live in as little as 50 μl of fluid for the first week, they readily absorb compounds from the water and are tolerant to DMSO making them ideally suited to screening compounds in 96-well plate format. Many zebrafish strains are available with fluorescent cells or organs and changes in fluorescence in response to applied compounds can be quantified making the assay amenable to automation. A recent example is the use of transgenic zebrafish embryos expressing GFP in the myocardium as a high-throughput assay to screen compounds for QT interval prolongation. Embryos placed in a 96-well plate were filmed by an automated scanning fluorescence microscope system and heart rate was calculated automatically. Drugs that prolong the QT interval in humans caused a dose-dependent decrease in heart rate, whereas control compounds had no effect (Burns et al., 2005). These automated techniques can be applied to zebrafish mutants with cardiac rhythm disturbances that serve as models of human heart disease as a high-throughput screen for drugs with antiarrythmic activity. Recently a semi-automated screen in microplate format for anticonvulsant activity has been published using a zebrafish model of epilepsy (Berghmans et al., 2007). An important part of the drug discovery process involves target validation. A common approach to knock down expression of a target protein requires microinjection of short interfering RNAs (siRNAs) or antisense morpholinos, which are resistant to nucleases. These are microinjected into embryos at the one cell stage and interrupt gene expression. In addition to being widely used in developmental studies morpholino antisense technology provides a rapid method of target validation in a vertebrate system and helps identify medically relevant genes whose protein products may become targets for future pharmaceuticals. The automated injection of genetic material into zebrafish embryos complements the use of this model system for developmental and toxicological studies, as well compound screening.

Automated injection of genetic material or other substances, such as sperm, into eggs or embryos of *Drosophila* or starfish is also possible using this technique. The use of different microplate formats is also possible, as the manufacturer for each microplate type supplies the distance between well centres and these can be directly entered in the program.

The system described here is a practical solution for intracellular or intranuclear injection of large cells such as laboratories using the *Xenopus* oocyte expression system or injection of zebrafish embryos. This is a cost and time effective alterative to manual injection and can be used by untrained personnel, requiring only a simple calibration procedure for each microplate. It

can also be envisaged that this system can be adapted for further applications such as *in vitro* fertilization.

Acknowledgments

Thanks to Franck Bontems for the GFP clone. This work was supported by a Swiss National Science Foundation award to R.C.H.

References

Berghmans S, Hunt J, Roach A, Goldsmith P. Zebrafish offer the potential for a primary screen to identify a wide variety of potential anticonvulsants. Epilepsy Res 2007;75:18–28.

Bertrand D, Cooper E, Valera S, Rungger D, Ballivet M. Electrophysiology of neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes following nuclear injection of genes or cDNA. Methods Neurosci 1991;4:174–83.

Brand M, Granato M, Nüsslein-Volhard C.Nüsslein-Volhard C, Dahm R, editors. Keeping and raising zebrafish. Oxford: Zebrafish Oxford University Press; 2002. p. 7–37.

Burns CG, Milan DJ, Grande EJ, Rottbauer W, MacRae CA, Fishman MC. High-throughput assay for small molecules that modulate zebrafish embryonic heart rate. Nat Chem Biol 2005;1:263–4.

Gamarnik AV, Andino R. Replication of poliovirus in *Xenopus* oocytes requires two human factors. EMBO J 1996;15:5988–98.

Gamarnik AV, Andino R. Exploring RNA virus replication in *Xenopus* oocytes. Methods Mol Biol 2006;322:367–78.

Gamarnik AV, Boddeker N, Andino R. Translation and replication of human rhinovirus type 14 and mengovirus in *Xenopus* oocytes. J Virol 2000;74:11983–7.

Gurdon JB, Lane CD, Woodland HR, Marbaix G. Use of frog eggs and oocytes for the study of messenger RNA and its translation in living cells. Nature 1971;233:177–82.

Gurdon JB, Lingrel JB, Marbaix G. Message stability in injected frog oocytes: long life of mammalian alpha and beta globin messages. J Mol Biol 1973;80:539–51.

Gurdon JB, Woodland HR, Lingrel JB. The translation of mammalian globin mRNA injected into fertilized eggs of *Xenopus laevis*. I. Message stability in development. Dev Biol 1974;39:125–33.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn 1995;203:253–310.

Laskey RA, Gurdon JB. The translation of viral RNAs in frog oocytes. Hamatol Bluttransfus 1974;14:272–4.

Laskey RA, Gurdon JB, Crawford LV. Translation of encephalomyocarditis viral RNA in oocytes of *Xenopus laevis*. Proc Natl Acad Sci USA 1972;69:3665–9.

Lewis TM, Harkness PC, Sivilotti LG, Colquhoun D, Millar NS. The ion channel properties of a rat recombinant neuronal nicotinic receptor are dependent on the host cell type. J Physiol 1997;505(Pt. 2):299–306.

Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. Nat Rev Genet 2007;8:353–67.

Marsal J, Tigyi G, Miledi R. Incorporation of acetylcholine receptors and Cl-channels in *Xenopus* oocytes injected with *Torpedo* electroplaque membranes. Proc Natl Acad Sci USA 1995;92:5224–8.

Miledi R, Parker I, Sumikawa K. Properties of acetylcholine receptors translated by cat muscle mRNA in *Xenopus* oocytes. EMBO J 1982a;1:1307–12.

Miledi R, Parker I, Sumikawa K. Synthesis of chick brain GABA receptors by frog oocytes. Proc R Soc Lond B: Biol Sci 1982b;216:509–15.

Miledi R, Parker I, Sumikawa K. Recording of single gamma-aminobutyrate- and acetylcholine-activated receptor channels translated by exogenous mRNA in *Xenopus* oocytes. Proc R Soc Lond B: Biol Sci 1983;218:481–4.

Miledi R, Eusebi F, Martinez-Torres A, Palma E, Trettel F. Expression of functional neurotransmitter receptors in *Xenopus* oocytes after injection of human brain membranes. Proc Natl Acad Sci USA 2002;99:13238–42.

Miledi R, Palma E, Eusebi F. Microtransplantation of neurotransmitter receptors from cells to *Xenopus* oocyte membranes: new procedure for ion channel studies. Methods Mol Biol 2006;322:347–55.

Morales A, Aleu J, Ivorra I, Ferragut JA, Gonzalez-Ros JM, Miledi R. Incorporation of reconstituted acetylcholine receptors from *Torpedo* into the *Xenopus* oocyte membrane. Proc Natl Acad Sci USA 1995;92:8468–72.

Palma E, Trettel F, Fucile S, Renzi M, Miledi R, Eusebi F. Microtransplantation of membranes from cultured cells to *Xenopus* oocytes: a method to study neurotransmitter receptors embedded in native lipids. Proc Natl Acad Sci USA 2003;100:2896–900.

Romero MF, Kanai Y, Gunshin H, Hediger MA. Expression cloning using *Xenopus laevis* oocytes. Methods Enzymol 1998;296:17–52.

Sakai T, Tochio H, Tenno T, Ito Y, Kokubo T, Hiroaki H, et al. In-cell NMR spectroscopy of proteins inside *Xenopus laevis* oocytes. J Biomol NMR 2006;36:179–88.

Selenko P, Wagner G. Looking into live cells with in-cell NMR spectroscopy. J Struct Biol 2007;158:244–53.

Selenko P, Serber Z, Gadea B, Ruderman J, Wagner G, Quantitative. NMR analysis of the protein G B1 domain in *Xenopus laevis* egg extracts and intact oocytes. Proc Natl Acad Sci USA 2006;103:11904–9.

Sivilotti LG, McNeil DK, Lewis TM, Nassar MA, Schoepfer R, Colquhoun D. Recombinant nicotinic receptors, expressed in *Xenopus* oocytes, do not resemble native rat sympathetic ganglion receptors in single-channel behaviour. J Physiol 1997;500(Pt. 1):123–38.

Westerfield M, editor. The zebrafish book: a guide for the laboratory use of zebrafish (*Danio rerio*). 4th ed. Eugene: University of Oregon Press; 2000.

Wickens MP, Woo S, O'Malley BW, Gurdon JB. Expression of a chicken chromosomal ovalbumin gene injected into frog oocyte nuclei. Nature 1980;285:628–34.

Woodland HR, Gurdon JB, Lingrel JB. The translation of mammalian globin mRNA injected into fertilized eggs of *Xenopus laevis*. II. The distribution of globin synthesis in different tissues. Dev Biol 1974;39:134–40.