Video Article

Xenopus Oocytes: Optimized Methods for Microinjection, Removal of Follicular Cell Layers, and Fast Solution Changes in Electrophysiological Experiments

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Abstract

The *Xenopus* oocyte as a heterologous expression system for proteins, was first described by Gurdon *et al.*[1] and has been widely used since its discovery (References 2 - 3, and references therein). A characteristic that makes the oocyte attractive for foreign channel expression is the poor abundance of endogenous ion channels[4]. This expression system has proven useful for the characterization of many proteins, among them ligand-gated ion channels.

The expression of $GABA_A$ receptors in *Xenopus* oocytes and their functional characterization is described here, including the isolation of oocytes, microinjections with cRNA, the removal of follicular cell layers, and fast solution changes in electrophysiological experiments. The procedures were optimized in this laboratory[5,6] and deviate from the ones routinely used[7-9]. Traditionally, denuded oocytes are prepared with a prolonged collagenase treatment of ovary lobes at RT, and these denuded oocytes are microinjected with mRNA. Using the optimized methods, diverse membrane proteins have been expressed and studied with this system, such as recombinant $GABA_A$ receptors[10-12], human recombinant chloride channels[13], Trypanosome potassium channels[14], and a *myo*-inositol transporter[15, 16].

The methods detailed here may be applied to the expression of any protein of choice in *Xenopus* oocytes, and the rapid solution change can be used to study other ligand-gated ion channels.

Video Link

The video component of this article can be found at https://www.jove.com/video/55034/

Introduction

Xenopus oocytes are widely used as an expression system (References 2 - 3, and references therein). They are able to properly assemble and incorporate functionally active multisubunit proteins into their plasma membranes. Using this system, it is possible to functionally investigate membrane proteins alone or in combination with other proteins, in order to study the properties of mutated, chimeric, or concatenated proteins, and to screen potential drugs.

Advantages of using oocytes over other heterologous expression systems include the simple handling of the giant cells, the high proportion of cells expressing foreign genetic information, the simple control of the environment of the oocyte by means of bath perfusion, and the control of the membrane potential.

The drawback of this expression system is the seasonal variation observed in many laboratories[17-20]. The reason for this variation is far from clear. Additionally, the quality of oocytes is often observed to vary strongly. Traditional methods[7-9] have included the isolation of ovary lobes, the exposure of ovary lobes to collagenase for some h, the selection of denuded oocytes, and the oocyte microinjection. Here, a number of alternative, fast procedures are reported that have allowed us to work with this expression system for more than 30 years with no seasonal variation and little variation in oocyte quality.

The modified and improved methods described here for the isolation of oocytes, microinjection with cRNA, and removal of follicular cell layers can be used for the expression of any protein of choice in the *Xenopus* oocyte. The very simple method for fast solution changes of the medium around the oocyte may be applied to the study of any ligand-gated ion channel and of carriers.

Protocol

Animal experiments have been approved by the local committee of the Canton Bern Kantonstierarzt, Kantonaler Veterinärdienst Bern (BE85/15).

1. Preparation of *Xenopus* Oocytes

1. Maintain frogs (*Xenopus laevis*) on a 12 h/12 h light/dark cycle in water that is strictly kept at 20 °C.
2. Remove the lobes of the ovaries from female frogs[9].
   NOTE: The removal of the lobes is a stimulus for regeneration, and often the quality of the oocytes improves with surgery. The oocyte is covered by a vitelline layer, a layer of follicle cells, and connective tissue containing the blood vessels[21]. The entire structure is termed the "follicle."
3. Place the lobes of the ovaries that are densely packed with the follicles containing the oocytes in sterile Barth's Saline[7] (MBS) supplemented with penicillin and streptomycin (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEPES (pH 7.5, NaOH), 100 µg/mL penicillin, and 100 µg/mL streptomycin).
4. Single-out stage V-VI[21] follicles. Hold the ovary lobe with forceps. Lower the platinum loop (**Figure 1**) over the follicles and gently withdraw the loop to disrupt the connective tissue containing the follicle from the ovary tissue.
   NOTE: Stage V-VI[21] follicles are characterized by their size (1.0 - 1.2 mm in diameter) and by the good contrast between the dark pigmented animal hemisphere and the yellowish vegetal hemisphere.
5. Transfer only healthy-looking follicles (*i.e.* perfectly spherical and with intact pigmentation) to a 35 mm diameter Petri dish by means of a plastic Pasteur pipette (cut the back to an opening diameter of 1.5 mm).

2. Microinjection of mRNA into the Cytoplasm

Note: The microinjection system described here is derived from that reported by Kressmann and Birnstiel[22].

1. Prepare cRNAs from the respective cDNAs coding for the subunits of the $\alpha_4\beta_2\delta$ $GABA_A$ receptor by *in vitro* transcription, the addition of a poly (A^+) tail, and RNA quantification by gel electrophoresis[23].
2. Prepare microinjection pipettes from borosilicate glass capillaries (1.0 mm outer diameter (OD), 0.58 mm inner diameter (ID), 100 mm length) using a micropipette puller. Break off the tips of the glass capillaries under a microscope using a micromanipulator and microfilament to create a tip diameter of 12 - 15 µm with a beveled tip.
3. Backfill the microinjection pipettes with paraffin oil using a 10 mL syringe, and then mount the microinjection pipette onto a homebuilt microinjection apparatus (**Figure 2**).
   NOTE: The homebuilt oil hydraulic injection apparatus consists of a grill motor controlled by a foot pedal switch. An additional switch changes the turning mode of the motor. This motor drives a micrometer screw (0.5 mm/turn) that advances or retracts the plunger of a 10 µL glass syringe. The injection is at a speed of about 3 rpm and a 0.5 turn corresponds to a 50 nL injection into a follicle. The tip of the syringe is connected to thick-walled polytetrafluoroethylene tubing that is itself connected to the injection needle by a very short piece of Tygon tubing. The injection capillary is held by a drill chuck that is controlled by a micromanipulator. The entire system is filled with paraffin oil. Any air bubbles should be avoided, as they will impair the injection system. The setup is lit with a cold light source. A stereomicroscope is required for optical control. Follicles are visualized under cold light with a stereomicroscope (40X magnification). Performance of the injection setup is tested by the injection of radioactive tracer into *Xenopus* oocytes. A volume of 45 - 55 nL should be delivered per injection.
4. Using a pipette with a sterile plastic tip, test the setup by placing a droplet of sterile water onto the clean inside of a moisture-resistant thermoplastic (2 x 2 cm). Immerse the tip of the injection pipette into this droplet, and then turn the motor to retract the plunger (negative pressure inside the injection pipette).
   NOTE: Water should enter the pipette and form a visible interface with the oil.
5. Retract the tip of the pipette from the droplet and apply positive pressure to the inside of the injection pipette. A water droplet should form at the tip of the injection pipette.
6. Prepare for the injection of the follicles by lining them up with the vegetal poles pointing upwards in the interstices of a nylon mesh (G: 0.8 mm) glued to the bottom of a Petri dish (60 mm) covered with MBS.
7. Dispense 2 µL of mRNA using a pipette with a sterile plastic tip onto the clean inside of a thermoplastic. Take the mRNA up into the injection pipette by applying negative pressure to the inside of the injection pipette.
8. Position the injection pipette over an individual follicle using the micromanipulator.
9. Insert the injection needle into the center of the vegetal pole and inject 50 nL of mRNA at a flow rate of 0.6 µL/min by applying positive pressure to the inside of the pipette. Wait 5 - 10 s before removing the injection pipette tip from the follicle to avoid mRNA escape.
   NOTE: The mRNA should be injected into the vegetal (yellowish) pole to avoid injection into the nucleus, which is located in the animal hemisphere.
10. Transfer the injected follicles to a new Petri dish (35 mm) filled with 2 mL of MBS. Place the dish into a wine cooler set at 18 °C. Incubate the injected follicles for 1 - 7 d before recording, depending on the identity of the newly expressed protein.

3. Stripping of the Follicles (Figure 3)

NOTE: As mentioned above, the oocyte covered by a vitelline layer, follicle cells, and connective tissue, which contains the blood vessels[24], is known as a "follicle." All layers except for the vitelline layer, which provides mechanical stability without preventing the access of solutions to the cell surface, must be removed before electrophysiological experiments. The follicle without the surrounding cell layers has previously been termed a "denuded" oocyte[25]. This step is usually performed on the same day as the electrophysiological experiments.

1. Transfer ten injected follicles to a borosilicate glass tube containing 0.5 mL of MBS, 1 mg/mL collagenase, and 0.1 mg/mL trypsin inhibitor. Immerse the tube in a water bath maintained at 36 °C. Incubate the follicles for 20 min and shake the tubes occasionally.
2. Rinse the follicles by transferring them to a second tube containing 1 mL of MBS at RT. Leave them in the tube for about 10 s.
3. Transfer the follicles to a third tube containing 0.5 mL of doubly concentrated MBS containing 4 mM Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and incubate them for 4 min at RT. Shake the tube occasionally.
   NOTE: The hypertonic solution (doubly concentrated MBS) is used to induce shrinkage of the oocytes within follicle cell layers, thereby detaching follicular cell layers from the oocyte; EGTA is added to inhibit collagenase activity.
4. Rinse the follicles by transferring them to a second tube containing 1 mL of MBS at RT. Leave them in the tube for about 10 s.
5. Transfer the oocytes to a Petri dish (35 mm diameter) containing 2 mL of MBS. Under a stereomicroscope, separate the outer envelopes from the follicles by simply pushing the naked oocyte away using the platinum loop.
   NOTE: The outer envelopes of the oocytes stick firmly to the culture dish and can be easily separated from the oocytes. Some oocytes will spontaneously lose their outer envelopes and will be recovered as denuded oocytes. This procedure results in a relatively stable preparation that keeps the spherical appearance of the oocytes.

4. Fast Solution Change around the Oocyte

1. Perform a voltage clamp experiment, as described[9,26].
2. Change the solution of the gravity-fed perfusion system by turning the perfusion switches as required by the experiment.
   1. Apply the perfusion solution at 6 mL/min through a glass capillary with an inner diameter of 1.35 mm, the mouth of which is placed about 0.4 mm from the surface of the oocyte, to allow fast changes in agonist concentration around the oocyte.
      NOTE: The rate of change has been previously estimated as 70% in less than 0.5 s[27].

Representative Results

Xenopus oocytes were mechanically singled out using a platinum loop (**Figure 1**). The oocytes were microinjected with mRNA coding for the $GABA_A$ receptor subunits α_4, β_2, δ, 0.5:0.5:2.5 fmol/oocyte (**Figure 2**). After 4 d, follicular cell layers were removed (**Figure 3**). Oocytes were voltage clamped at -80 mV and exposed to increasing concentrations of γ-aminobutyric acid (GABA) in the presence of 1 µM 3α,21-dihydroxy-5α-pregnan-20-one (THDOC), a potent positive allosteric modulator of the $GABA_A$ receptor. **Figure 4A** shows original current traces recorded in such an experiment. **Figure 4B** shows elicited current amplitude depending on the GABA concentrations. This allows determination of the sensitivity of this subunit combination toward GABA. The individual curves were fitted and standardized to I_{max} and subsequently averaged. The equation used was $I(c)=I_{max}/(1+(EC_{50}/c)^n)$, where c is the concentration of GABA, EC_{50} the concentration of GABA (in the presence of 1 µM THDOC) eliciting a half-maximal current amplitude, I_{max} is the maximal current amplitude, I is the current amplitude, and n is the Hill coefficient. The EC_{50} of the $\alpha_4\beta_2\delta$ $GABA_A$ receptor amounted to 0.41 ± 0.12 µM, and n was 0.76 ± 0.04.

Figure 1: Platinum Loop. The platinum wire loop mentioned in protocol step 1.4. Platinum is a metal that can be bent without producing splinters, and it does not stick to biological material. Please click here to view a larger version of this figure.

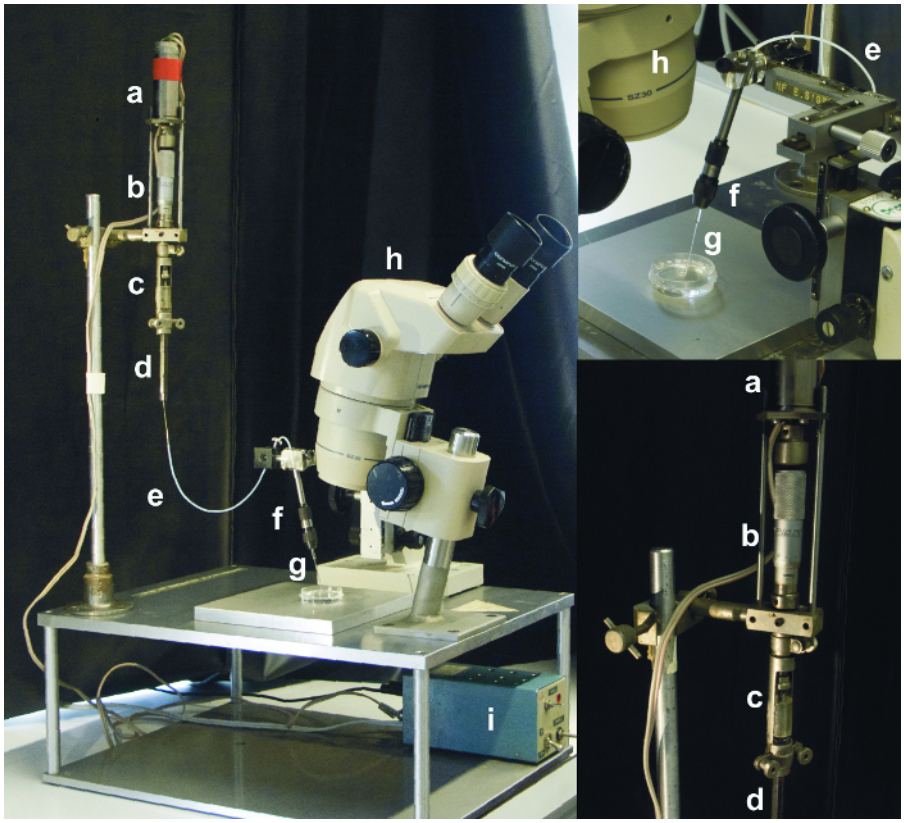

Figure 2: Homebuilt Microinjection Apparatus. The apparatus is described in protocol step 2. **a**, grill motor; **b**, micrometer screw; **c**, spring between syringe and plunger; **d**, 10 µL glass syringe; **e**, thick-walled polytetrafluoroethylene tubing; **f**, hand drill chuck; **g**, injection pipette; **h**, stereomicroscope; and **i**, motor control. Please click here to view a larger version of this figure.

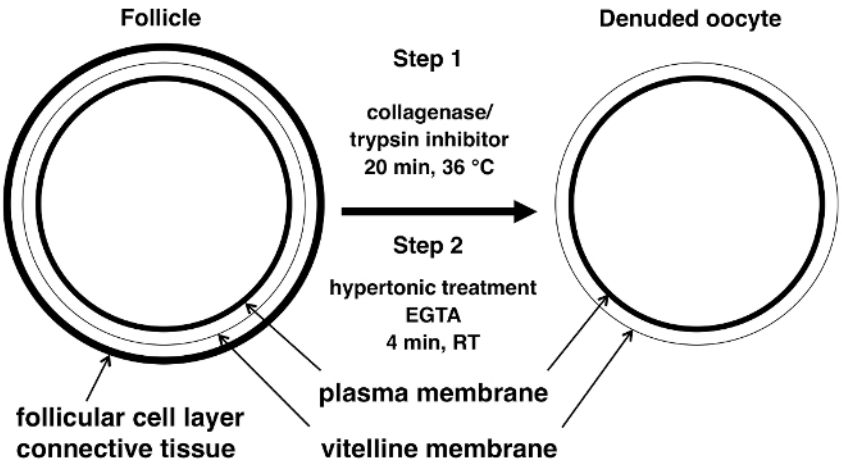

Figure 3. Scheme Showing the Defolliculation of an Oocyte. Oocytes are first incubated in a collagenase solution in a water bath at 36 °C for 20 min. Oocytes are then washed in a modified Barth medium. The final incubation step is in a hypertonic EGTA solution at RT for 4 min. As a result, the connective tissue and the follicle cells are removed to give way to the denuded oocyte. Please click here to view a larger version of this figure.

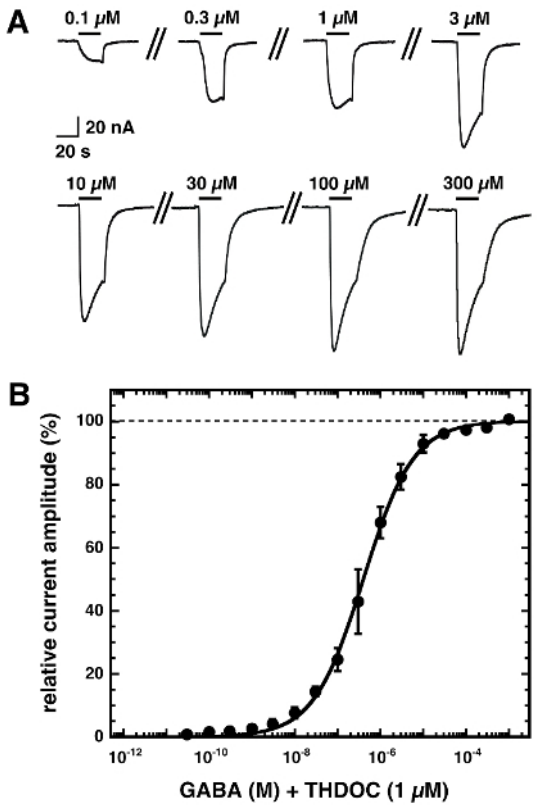

Figure 4. Current Traces from a Two-electrode Voltage Clamp Experiment. A, Current traces from a GABA concentration response curve in the presence of 1 μM THDOC obtained from a *Xenopus* oocyte expressing the $\alpha_4\beta_2\delta$ $GABA_A$ receptor. The bars indicate the time period of GABA/1 μM THDOC perfusion. Increasing concentrations of GABA were applied to the oocytes, and the corresponding current amplitudes were determined. GABA concentrations are indicated above the bars. **B**, Averaged concentration response curves of the $\alpha_4\beta_2\delta$ $GABA_A$ receptor. Individual curves were first normalized to the fitted maximal current amplitude and were subsequently averaged. The data are shown as mean ± SD, *n* = 3. Please click here to view a larger version of this figure.

Discussion

The methods described in this article deviate from those used traditionally[7-9]. It is standard to expose the lobes of the ovary to a 1 to 2 h collagenase treatment[8]; isolate undamaged, denuded oocytes; and inject them with mRNA using commercial injection devices. This classical procedure has the following drawbacks: 1) Oocytes are likely to be damaged by the long exposure to high concentrations of collagenase. 2) The unstable denuded oocytes must be stored until the experiment. 3) Denuded oocytes are more likely to suffer during microinjection than follicles. Commercial injection devices use large-diameter (about 20 μm) injection needles, likely to result in a relatively high rate of damage. The advantages of the improved procedure described here are as follows: exposure to collagenase is limited to 20 min, denuded oocytes do not have to be stored, and the small tip diameter of the pipettes (about 12 - 15 μm) used for microinjection does not require prior "defolliculation" of the follicles for the mRNA injection. The single critical step is that the follicle incubation temperature in collagenase solution should be carefully adjusted to 36 °C and should not exceed it.

For solution changes in electrophysiological experiments, very often the solution in the measurement chamber is changed, which takes a substantial amount of time, depending upon size of the chamber and the perfusion rate. Using the perfusion capillary avoided this.

The major drawback of the expression of ion channels in *Xenopus* oocytes is their giant size. Voltage control faster than about 2 ms is difficult, and very fast (<0.5 s) solution changes require elaborate procedures. Furthermore, strongly hydrophobic substances may almost be irreversibly bound to the egg yolk of the oocyte.

The methods outlined here have allowed us to investigate in a time-saving manner the functional properties of recombinant $GABA_A$ receptors, their modulation by synthetic compounds and plant substances, their subunit arrangement, and the location of drug binding sites in receptors of different subunit composition. The methods described here are suited for the expression of any protein of choice in the *Xenopus* oocyte. The very simple method for fast solution changes of the medium around the oocyte may be applied to the study of any ligand-gated ion channel or carrier.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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