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TITLE:

Recording Gap Junction Current from *Xenopus* Oocytes

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KEYWORDS

gap junction; innexin; *Caenorhabditis elegans*; *C. elegans*; *Xenopus* oocytes; junctional current; voltage clamp

SUMMARY:

Here we present a protocol to express gap junction proteins in *Xenopus* oocytes and record junctional current between two apposed oocytes using a commercial amplifier designed for dual oocyte voltage-clamp recordings in a high side current measuring mode.

ABSTRACT:

Heterologous expression of connexins and innexins in *Xenopus* oocytes is a powerful approach for studying the biophysical properties of gap junctions (GJs). However, this approach is technically challenging because it requires a differential voltage clamp of two opposed oocytes sharing a common ground. Although a small number of labs have succeeded in performing this technique, essentially all of them have used either homemade amplifiers or commercial amplifiers that were designed for single-oocyte recordings. It is often challenging for other labs to implement this technique. Although a high side current measuring mode has been incorporated into a commercial amplifier for dual oocyte voltage-clamp recordings, there had been no report for its application until our recent study. We have made the high side current measuring approach more practical and convenient by introducing several technical modifications, including the construction of a magnetically based recording platform that allows precise placement of oocytes and various electrodes, use of the bath solution as a conductor in voltage differential electrodes, adoption of a commercial low-leakage KCl electrode as the reference electrode, fabrication of current and voltage electrodes from thin-wall glass capillaries, and positioning of all the electrodes using magnetically based devices. The method described here allows convenient and robust recordings of junctional current (I_j) between two opposed *Xenopus* oocytes.

INTRODUCTION

GJs are intercellular channels that may allow the current flow and exchange of small cytosolic molecules between neighboring cells. They exist in many cell types and perform diverse physiological functions. GJs in vertebrates are formed by connexins, whereas those in invertebrates by innexins. Each GJ consists of two juxtaposed hemichannels with either 6 or 8 subunits per hemichannel, depending on whether they are connexins or innexins[1–3]. Humans have 21 connexin genes[4], while the commonly used invertebrate models *C. elegans* and *Drosophila melanogaster* have 25 and 8 innexin genes, respectively[5,6]. Alternative splicing of gene transcripts may further increase the diversity of GJ proteins, at least for innexins[7,8].

GJs may be divided into three categories based on molecular compositions: homotypic, heterotypic, and heteromeric. A homotypic GJ has all its subunits being identical. A heterotypic GJ has two homomeric hemichannels, but the two hemichannels are formed by two different GJ proteins. A heteromeric GJ contains at least one heteromeric hemichannel. The molecular diversities of GJs may confer distinct biophysical properties that are important to their physiological functions. GJ biophysical properties are also modulated by regulatory proteins[9]. To understand how GJs perform their physiological functions, it is important to know their molecular compositions, biophysical properties, and the roles of regulatory proteins in their functions.

Heterologous expression systems are often used to study biophysical properties of ion channels, including GJs, and the effects of regulatory proteins on them. Because heterologous expression systems allow the expression of specific proteins, they are generally more amenable to dissecting protein functions than native tissues where proteins with redundant functions can complicate the analysis, and recording of I_j can be unattainable. Unfortunately, most commonly used cell lines except the Neuro-2A cell are inappropriate for studying GJ biophysical properties due to complications by endogenous connexins. Even Neuro-2A cells are not always appropriate for this kind of analysis. For example, we could not detect any I_j in Neuro-2A cells transfected with the innexins UNC-7 and UNC-9 in either the absence or the presence of UNC-1 (unpublished), which is required for the function of UNC-9 GJs in *C. elegans*[9,10]. On the other hand, *Xenopus* oocytes are a useful alternative system for electrophysiological analyses of GJs. Although they express an endogenous GJ protein, connexin 38 (Cx38)[11], potential complications can be easily avoided by injecting a specific antisense oligonucleotide[12]. However, analyses of GJs with *Xenopus* oocytes require a differential voltage clamp of two juxtaposed cells, which is technically challenging. The earliest successes of double voltage clamp of frog blastomeres were reported about 40 years ago[13,14]. Since then, many studies have used this technique to record I_j in paired *Xenopus* oocytes. However, essentially all the previous studies have been performed with either homemade amplifiers[12,15,16] or commercial amplifiers designed for recordings on single oocytes (GeneClamp 500, AxoClamp 2A, or AxoClamp 2B, Axon Instruments, Union City, CA)[8,17–20]. Because even the commercial amplifiers do not provide instructions for double oocyte voltage clamp, it is often challenging for new or less sophisticated electrophysiological labs to implement this technique.

Only one commercial amplifier has been developed for double oocyte voltage clamp, the OC-725C from Warner Instruments (**Table of Materials**, **Figure 1A**). This amplifier may be used in either a standard mode (for single oocytes) or a high side current measuring mode (for single or

dual oocytes) depending on whether two sockets in its voltage probe are connected (**Figure 1B, C**). However, until our recent study[7], there had not been a single publication describing the use of this amplifier in its high side current measuring mode. Although the amplifier has been used by another lab for dual oocyte recordings, it was used in the standard rather than the high side mode[21,22]. This lack of reports using the amplifier in its high side current measuring mode might be due to technical difficulties. We were unable to obtain stable dual oocyte recordings using the high side mode by following instructions from the manufacturer. Over the years, we have tried three different approaches for dual oocyte recordings, including using two OC-725C amplifiers in the high side current measuring mode, two OC-725C amplifiers in the standard mode, and two amplifiers from another manufacturer. We eventually succeeded in obtaining stable recordings only with the first approach after extensive trial and error. This publication describes and demonstrates the procedures we use to express GJ proteins in *Xenopus* oocytes, record I_j using the high side current measuring mode, and analyze the electrophysiological data using popular commercial software. Additional information about the double voltage-clamp technique may be found in other publications[19,23].

PROTOCOL:

The surgeries are performed following a protocol approved by the institutional animal care committee of the University of Connecticut School of Medicine.

1. Frog surgery and preparation of defollicuated oocytes

1.1 Anesthetize an adult female African clawed frog (*Xenopus laevis*) (**Table of Materials**) by immersing in a cool (with ice) tricaine solution (~300 mg/L).

1.2 Wait (~15 min) until the frog shows little or no response to squeezing its webbed feet. Lay the frog on an operating table with its belly facing up.

1.3 After a longitudinal incision (8–10 mm long) on either the left or the right lower abdominal region, gently pull out a small piece of ovary tissue with a pair of forceps, and cut free the ovary tissue with a pair of small scissors.

1.4 Immediately immerse the free ovary tissue in a Ca^{2+}-free ND96 solution (NaCl 99 mM, KCl 2 mM, $MgCl_2$ 1 mM, HEPES 5 mM, pH 7.5) inside a 60-mm Petri dish.

1.5 After closing the incision by simple interrupted sutures using a 5-0 silk suture (**Table of Materials**), put the frog back into a shallow water tank for recovery.

NOTE: The incision is closed in two steps: first the peritoneal and muscle layers, and then the skin layer. Each frog is subjected to 5 surgeries with at least a 4-week interval between two consecutive surgeries.

1.6 Transfer the isolated ovary tissue to a 50-mL centrifuge tube containing 10 mL of Ca^{2+}-

free ND96 solution with 20 mg of collagenase (**Table of Materials**) and 20 mg of hyaluronidase (**Table of Materials**).

1.7 Shake the tube on an orbital shaker at room temperature (RT) until all the oocytes have become isolated (solitary).

1.8 Thereafter, transfer 30–50 oocytes to a Petri dish using a glass Pasteur pipette and examine the oocytes frequently under a stereomicroscope (≥25x highest magnification power) to determine whether they are defolliculated.

NOTE: The Pasteur pipette should be "cut" to a wider tip opening using a diamond scriber (**Table of Materials**) and flamed to smooth the cutting edge.

1.9 As soon as 70%–80% of the oocytes are defolliculated, decant the enzyme solution by gently tilting the tube. Wash the oocytes 5 times by filling up the tube with ND96 solution (NaCl 96 mM, KCl 2 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, HEPES 5 mM, pH 7.5) and decanting the solution.

1.10 After the final wash, transfer the oocytes to a 60-mm Petri dish containing ND96 solution. Pick and transfer large and healthy-looking oocytes to a 60-mm Petri dish containing ND96 solution supplemented with sodium pyruvate (2 mM) and penicillin-streptomycin (100 U/mL) using a clean glass Pasteur pipette.

NOTE: "large and healthy-looking oocytes" are those of stages V and VI showing no sign of over-digestion by the enzymes.

1.11 Place the Petri dish containing the picked oocytes inside an environmental chamber (15–18 °C).

2. GJ protein expression

2.1 Synthesize complementary RNA (cRNA) of a specific connexin or innexin *in vitro* using an RNA transcription kit (**Table of Materials**) following the manufacturer's protocol.

2.2 Precipitate the cRNA using a lithium chloride method described in the user manual of the RNA transcription kit.

2.3 Wash the pellet with 70% ethanol, dissolve it in 20 µL of nuclease-free H_2O, and add 1 µL of ribonuclease inhibitor (40 U, **Table of Materials**).

2.4 Measure the concentration of the cRNA using a spectrophotometer (**Table of Materials**).

2.5 Mix the cRNA with a Cx38 antisense oligo so that the final concentrations are 200–1,000 ng/µL cRNA and 100 ng/µL oligo.

NOTE: The oligo sequence is 5'-GCTTTAGTAATTCCCATCCTGCCATGTTTC-3', which corresponds to the nucleotides from -5 to +25 in *Xenopus laevis* Cx38 mRNA (NCBI Accession: NM_001088018)[11]. The oligo is kept as a stock solution (2.0 mg/mL) prior to being mixed with the cRNA.

2.6 Eliminate particles in the cRNA by spinning in a microcentrifuge (~16,000 x *g*) for 2 min, and quickly transfer the entire supernatant to a new tube by pipetting (avoid disturbing the bottom of the microcentrifuge tube).

2.7 Divide the cRNA into aliquots (2.5 µL/vial), and store the aliquots in a -80 °C freezer.

2.8 Prepare an oocyte injection dish by gluing a small piece (~1 cm x 1 cm) of nylon mesh (**Table of Materials**) to the bottom of a 35-mm Petri dish using a quick-cure epoxy adhesive.

NOTE: The oocyte injection dish is reusable. Wash it with 70% ethanol after each use.

2.9 Fill the oocyte injection dish approximately halfway with ND96 solution (supplemented with pyruvate and penicillin-streptomycin).

2.10 Place 25–30 oocytes in rows inside the Petri dish.

2.11 Prepare glass micropipettes for oocyte injections by following instructions in the user manual of the automated nanoliter injector (**Table of Materials**).

NOTE: Use of a microelectrode beveler (**Table of Materials**) can help produce sharp injection micropipettes that cause minimum damage to oocytes.

2.12 Backfill an injection micropipette with lightweight mineral oil, and insert it into the micropipette holder of the injector.

2.13 Transfer the cRNA from one of the stored aliquots to the inside surface of a Petri dish cover or bottom by pipetting.

2.14 Aspirate the cRNA droplet into the tip of the micropipette by pressing the **FILL** button in the injector controller.

2.15 Inject the cRNA (~50 nL/oocyte) by pressing the **INJECT** button.

NOTE: The injection volume may be set by the dip switches in the injector controller.

2.16 Transfer the injected oocytes to a new Petri dish containing ND96 solution (supplemented with pyruvate and penicillin-streptomycin), and keep them inside the environmental chamber (15–18 °C) for 1–3 days (depending on GJ protein expression speed and level). Replace the solution daily by transferring the oocytes to a new Petri dish.

3. Oocyte pairing

3.1 Transfer a few of the injected oocytes into a 35-mm Petri dish containing ND96 solution.

3.2 Use two fine tweezers (**Table of Materials**) to gently peel off the transparent vitelline membrane that wraps the oocyte.

NOTE: It may be necessary to sharpen the tweezer tips before the first use and from time to time, using a piece of fine (600 Grit) sandpaper.

3.3 Place 2 oocytes per well in an oocyte pairing chamber (**Figure 1D**) containing ND96 solution (supplemented with pyruvate and penicillin-streptomycin).

NOTE: The oocyte pairing chamber is constructed by gluing a small piece of a Microwell Minitray (**Table of Materials**) to the bottom of a 35-mm petri dish (**Table of Materials**) using a quick-cure epoxy adhesive. The Minitray is cut into small pieces using a hot wire cutter (**Table of Materials**). Use of the Minitray for oocyte pairing was originally described by others[24]. Although a GJ protein might distribute non-uniformly in the oocyte cell membrane depending on charge properties of amino acid residues in its cytosolic domains[25], random pairing (without discriminating between the animal and vegetal poles of the oocyte) appears to be sufficient in general.

3.4 Keep the Petri dish containing paired oocytes at RT on the isolation table to be used for electrophysiological recordings.

NOTE: The pairing duration can vary from a few hours to one day. A convenient practice is to pair oocytes in the late afternoon and record them on the following day.

4. Acquisition system preparation

4.1 Configure two OC-725C oocyte clamp amplifiers (**Table of Materials**) to the high side current measuring mode by adjusting an internal dip switch (**Figure 1B**).

4.2 Ground the amplifiers by first interconnecting the Ground Circuit sockets on the rear panels and then connecting to the Faraday cage and the fiber light used for illuminating the recording chamber.

4.3 Connect the amplifiers to an analog-to-digital signal converter (**Table of Materials**), and configure them in the Clampex module of the pClamp software (**Table of Materials**) following instructions from the amplifier manufacturer.

4.4 Test the acquisition system with the model cell supplied with the amplifier.

4.4.1 Connect the V_{DIFF} probe and current (I) cables to the model cell, connect the Red and Black sockets in the V_{DIFF} probe with the included jumper, connect either leg of the jumper to either

pin in the model cell, and connect the ground wire in the model cell to the cable from the **GROUNDS CIRCUIT** socket of the amplifier (**Figure 1E**).

4.4.2 Zero the Voltage Electrode (Vm) and Bath Electrode (Im) meters of the amplifier by turning the **Vm OFFSET** and **Ve OFFSET** knobs, respectively, switch Clamp from **OFF** to either **Fast** or **SLOW** and turn the **GAIN** dial clockwise to a level that allows proper voltage clamp (**Figure 1A**). The D.C. GAIN switch may be at either the **OUT** or the **IN** position.

4.4.3 Run a simple acquisition protocol containing a few voltage steps to confirm that the voltage displayed in the Vm meter changes according to the acquisition protocol and that voltage and current traces are displayed properly in Clampex.

NOTE: Only one amplifier may be tested each time using this approach.

5. Recording I_j between paired oocytes

5.1 Create acquisition protocols for recording I_j in the pClamp software.

NOTE: Create two acquisition protocols. In one of them, amplifier # 1 is used to produce a series of membrane voltage (V_m) steps (e.g., -150 to +90 mV at 10-mV intervals), whereas amplifier #2 is used to maintain a constant V_m (e.g., -30 mV). In the other protocol, amplifier #2 is used to produce the V_m steps, whereas amplifier # 1 is used to maintain the constant V_m. The positive and negative V_m steps should be applied alternatively (e.g., -150, +90, -140, +80 ……), which can be programmed in Clampex using the **User List** feature in the acquisition protocol.

5.2 Set up the recording stage

5.2.1 Drop one Petri dish containing paired oocytes into the Petri dish receptacle in the recording platform (**Figure 2A**)

5.2.2 Select a pair of oocytes and rotate the Petri dish if necessary so that the two oocytes are in the left and right direction, and lock the stage in position by its magnetic base.

5.2.3 Fix the magnetic stands holding the current and voltage probes at appropriate locations on the isolation table.

NOTE: Make sure that the current and voltage probes from one amplifier are located on the left side, whereas those from the other amplifier are on the right side, and that the voltage probe is in front of the current probe on each side (**Figure 2B**,**C**).

5.3 Set up the reference electrode

5.3.1 Place a reference electrode (**Table of Materials**) near the edge of the Petri dish toward the user side (**Figure 2B**,**C**).

5.3.2 Connect the reference electrode to the black socket (Circuit Ground) in only one of the two V_{DIFF} probes.

5.4 Set up the V_{DIFF} electrodes

5.4.1 Pull a pair of glass micropipettes, break off a bit of the tip with the diamond scriber (**Table of Materials**), and smooth the tip edge by fire polishing.

NOTE: Tip resistance should be less than 150 kΩ (measured with ND96 in the pipette). Typically micropipettes with tip resistance of 20–150 kΩ are used.

5.4.2 Hold the micropipette above the flame of an alcohol burner to bend it to a smooth angle (~130°) at a position ~1 cm away from the tip.

NOTE: Fabricated micropipettes are reusable. Rinse them with water after each use.

5.4.3 Backfill the glass pipette completely with ND96 solution, insert it into a prefilled (with ND96) microelectrode holder (**Table of Materials**, **Figure 2D**), and ensure no air bubbles are present in the system.

5.4.4 Insert the 2-mm pin of the microelectrode holder into the 2-mm socket of a V_{DIFF} electrode connection wire (**Figure 2D**).

5.4.5 Clamp the 2-mm socket on a magnetically based clamper (**Figure 2D**), and aim the tip of the V_{DIFF} electrode toward one of the two oocytes (**Figure 2E**).

NOTE: Adjust the position and angle of the clamper so that the tip of the electrode is very close to the oocyte

5.4.6 Insert the 1-mm pin of the V_{DIFF} electrode connection wire into the red socket (V_{DIFF} Input) in the V_{DIFF} probe on the same side.

5.4.7 Prepare and connect a V_{DIFF} electrode for the other oocyte and amplifier following similar procedures.

NOTE: A close-up view of a pair of oocytes and all the electrodes are shown in **Figure 2E**.

5.5 Set up the current and voltage electrodes

5.5.1 Prepare voltage and current electrodes from glass capillaries, backfill them (about halfway) with a KCl solution (KCl 3.0 M, EGTA 10 mM, HEPES 10 mM, pH 7.4 with KOH), and insert them into the electrode holders provided with the amplifiers.

NOTE: The electrodes should have a resistance of ~1 MΩ. Suitable electrodes may be obtained from a type of thin-wall glass capillaries (**Table of Materials**) using a specific set of pulling parameters (**Table 1**). Such tips can easily penetrate the oocyte cell membrane without the need to press either the Vm or the Ve BUZZ button on the amplifier.

5.5.2 Lower the electrodes into the bath solution, zero the Vm and Im meters by turning the **Vm OFFSET** and **Ve OFFSET** dials and check electrode resistance by pressing **Vm Electrode Test** and **Ve Electrode Test**.

5.5.3 Insert the current and voltage electrodes into the oocytes, and observe negative membrane potentials (typically -20 to -50 mV).

NOTE: The Vm and Im meters of the same amplifier should display two identical or very similar values.

5.6 Data acquisition

5.6.1 In the Clamp section of the amplifier, confirm that D.C. GAIN is at the **IN** position, turn the **GAIN** knob clockwise to a level that allows proper voltage clamp (typically one-third to half of the full range), and turn the Clamp switch from **OFF** to **FAST**.

NOTE: Upon turning on the clamp, the Vm and Im meters will display the holding voltage and the holding current, respectively. Although the holding current may vary somewhat depending on oocyte conditions, it is generally small and stable at the holding V_m (e.g., -30 mV).

5.6.2 Run an acquisition protocol.

NOTE: Four traces will be displayed on the screen. The voltage and current traces from one amplifier indicate the *Vm* steps applied to oocyte #1 and the current needed to produce the *Vm* steps, whereas those from the other amplifier indicate the constant *Vm* (-30 mV) of oocyte #2, and the current injected to maintain this constant *Vm*. The current injected into oocyte #2 represents the I_j.

6. Data analysis

6.1 Analysis with Clampfit.

6.1.1 Open a recorded *abf* file in the Clampfit module of pClamp.

6.1.2 Use cursors 1 and 2 to enclose a segment of the baseline before the I_j traces.

6.1.3 Click on the icon **Adjust Baseline** to bring out a Baseline window. Select **Subtract Mean of Cursors 1..2** for Method, and confirm that **All Visible Signals** and **All Visible Traces** are selected for Trace Selection.

6.1.4 Upon clicking **OK**, the Baseline window closes, the baselines of all the current and voltage traces converge at the zero level. Note that the voltage steps change to new levels that are equal to the original voltage minus the holding voltage (e.g., -30 mV).

6.1.5 To plot the I_j and V_j relationship using steady-state I_j, enclose a desired segment of the I_j traces representing steady-state I_j with cursors 1 and 2, place cursor 3 anywhere within the time window of the voltage steps.

6.1.6 Go to **Analyze**/**Quick Graph**/**I-V** to open an *I-V* window.

6.1.7 Under **X-Axis** (Voltage), select **Cursor 3 from Signal**, specify the voltage step signal (e.g., Voltage 1) in the dropdown menu, and check the box **Invert.**

NOTE: The Invert box is checked to convert the V_m to values equivalent to V_j, which is defined as V_m of oocyte #2 – V_m of oocyte #1.

6.1.8 Under **Y-Axis** (Current), select the source of the I_j signal (e. g., *Current 0*) from the dropdown menu next to **Signal**, define **Region** as **Cursors 1..2** from the dropdown menu, and select **Mean**.

NOTE: To plot peak I_j and V_j relationships, cursors 1 and 2 should enclose the segment of I_j traces containing the peak I_j in step 6.1.5 and select **Peak** (instead of **Mean**) in step 6.1.8.

6.1.9 Under **Destination** option, select either **Replace** or **Append**.

6.1.10 Upon clicking **OK** in the *I-V* window, an I_j - V_j relationship is displayed on the screen, and the corresponding I_j and V_j values may be found by clicking **Window/Result**.

6.2 Plot and fit the G_j – V_j relationship in Origin (**Table of Materials**)

6.2.1 Copy the two columns containing V_j and I_j values from the Results window of Clampfit (step 6.1.10) to a new Workbook in Origin. Make sure that the V_j and I_j values are under X and Y columns, respectively.

6.2.2 Add four new columns in the Workbook by clicking **Column**/**Add New Columns**.

6.2.3 Name the first new column as G_j, fill it by entering the equation "column I_j/column V_j * 1,000" in the **Set Column Values** window, and create a scatter plot of the G_j – V_j relationship.

NOTE: The multiplication by 1,000 (optional) is to avoid the inconvenience of dealing with very small numbers in subsequent steps.

6.2.4 Fit the G_j data points over the negative and positive V_j ranges independently to the

Boltzmann function. Calculate G_j at V_j = 0 mV by entering the G_{jmax}, G_{jmin}, A, and V_0 values from the fitting into the Boltzmann equation, which can be done in a spreadsheet. The G_j thus obtained will be used as G_{jmax} in the next step.

NOTE: The equation to fit the Boltzmann function is: $G_j = (G_{jmax} – G_{jmin})/\{1 + \exp[A(V_j – V_0)]\} + G_{jmin}$, in which V_0 is the V_j at which the conductance is half-maximal, G_{jmax} is the maximal conductance, G_{jmin} is the V_j-insensitive residual conductance[23]. To perform the fitting, first add the Boltzmann equation as a new fitting function into Origin, and then select this function for fitting. Enter approximate seed values for the G_{jmax}, G_{jmin}, V_0, and A before executing the fitting function. Make sure that the G_{jmax} parameter is not fixed for this fitting.

6.2.5 Name the second and third new columns as nGj-L and nGj-R ("n" for "normalized"), and fill them by dividing the G_j column by the G_{jmax} values derived from the left and right G_j - V_j curves, respectively (step 6.2.4).

6.2.6 Name the fourth new column as nGj-LR, and fill it by copying values from the negative and positive V_j ranges of the nGj-L and nGj-R columns, respectively.

6.2.7 Create a scatter plot for nGj-LR over V_j, and fit the data points over the negative and positive V_j ranges to the Boltzmann function independently. Make sure that the G_{jmax} parameter is fixed at 1.0 for the fitting.

6.2.8 Keep records of the fitting results (e.g., the fitted graph and the G_{jmin}, V_0, and A values).

REPRESENTATIVE RESULTS:

UNC-7 and UNC-9 are innexins of *C. elegans*. While UNC-9 has only one isoform, UNC-7 has multiple isoforms that differ mainly in the length and amino acid sequence of their amino terminals[7,8]. These innexins may form homotypic as well as heterotypic (of UNC-7 and UNC-9) GJs when expressed in *Xenopus* oocytes[7,8]. Representative I_j traces and the resulting normalized $G_j – V_j$ relationships of UNC-7b and UNC-9 homotypic GJs are shown in **Figure 3**. In these experiments with paired oocytes, V_m of Oocyte 1 were clamped to different levels from the holding voltage (-30 mV), whereas that of Oocyte 2 was kept constant at -30 mV to monitor the I_j. The results show that these two types of GJs differ in the V_j-dependent I_j inactivation rate, V_j dependence (indicated by the slope of the $G_j – V_j$ curve), and the amount of the residual G_j. Many other examples of UNC-7 and UNC-9 GJs, including rectifying GJs, may be found in our recent publication[7].

FIGURE AND TABLE LEGENDS:

Figure 1: Oocyte pairing chamber and amplifier setup. (**A**) Front panel of the oocyte clamp amplifier OC-725C. (**B**) A DIP switch inside the amplifier configured for the high side current measurement. All the toggle switches except 2, 5, and 7 are in the OFF position to use the amplifier in the high side current measuring mode. (**C**) A V_{DIFF} probe with the red socket (for V_{DIFF} input) and black socket (for Circuit Ground) either unconnected or connected. The probe may be used for the standard voltage-clamp mode when the two sockets are connected. (**D**) An oocyte

pairing chamber. (**E**) A model cell with connections for testing the acquisition system with the amplifier in the high side current measuring mode.

Figure 2: Oocyte and electrode setup. (**A**) The recording stage. The circular hole has a diameter of 36 mm. (**B**) Diagram showing the positions of the various electrodes. (**C**) Actual layout of the various electrodes. (**D**) Two V_{DIFF} electrodes with their holders and connection cables clamped on the recording stage by two different magnetic clamps, including a modified Agar Bridge Magnetic Holder (**Table of Materials**) (top) and a tube clamp (**Table of Materials**) (bottom). The former is more stable in maintaining the electrode position because of its larger magnetic base but requires modification. The glass micropipettes are rotated 90° from their operating positions in order to show the bending angles. (**E**) A close-up view of a pair of oocytes and the electrodes.

Figure 3: Representative recording traces. (**A**) Diagram showing the oocyte experiment. Negative and positive membrane voltage (V_m) steps are applied to Oocyte 1 from a holding V_m of -30 mV whereas Oocyte 2 is held at a constant V_m of -30 mV. The transjunctional voltage (V_j) is defined as V_m of Oocyte 2 – V_m of Oocyte 1. (**B)**. Sample I_j traces and the resulting normalized junctional conductance (G_j) – V_j relationship of UNC-9 homotypic gap junctions. (**C**) Sample I_j traces and the resulting normalized G_j-V_j relationship of UNC-7b homotypic gap junctions. The G_j-V_j relationships are fitted by a Boltzmann function (red lines).

Table 1: Electrode pulling parameters. These parameters are based on thin-wall glass capillaries (**Table of Materials**) and a ramp temperature of 258 at a P-97 micropipette puller (**Table of Materials**). They need to be adjusted according to the ramp temperature for this glass on your puller. For example, if the ramp temperature on the puller is 20° higher, add 20° to each step and make necessary adjustments. Generally, tip size may be optimized by adjusting the velocity of the last step. Please refer to the user manual at the manufacturer's website for meanings of the pulling parameters (https://www.sutter.com/MICROPIPETTE/p-97.html).

DISCUSSION:

System optimization appears to be necessary for dual oocyte voltage-clamp experiments. Without it, recordings can be highly unstable, and the amplifiers may have to inject an excessive amount of current to reach the target *Vm*, resulting in oocyte damage and recording failures. Several factors are critical to obtaining stable dual oocyte recordings with the high side current measuring method. First, the current and voltage electrodes must have appropriate resistance (~1 MΩ), and their holders must be clean. Second, the V_{DIFF} electrodes must have low resistance (<150 kΩ) and be close to the oocytes. Third, all the electrodes for the same oocyte (voltage, current, and V_{DIFF}) must be positioned on the same side (left or right), and the order of the electrodes (from back to front) should be current, voltage, and V_{DIFF}. Lastly, the reference electrode should be located near the edge of the 35-mm Petri dish toward the user.

We have modified a few recommended procedures from the manufacturer and made some other improvements. Among them are: 1) ND96-filled micropipettes instead of KCl-loaded agar bridges to serve as V_{DIFF} electrodes. The glass electrodes are easy to construct, reusable, and non-harmful to oocytes; 2) a low leakage KCl electrode as the reference electrode. This electrode has low

resistance (~2.7 kΩ) and stable potential, and leaks little electrolytes (~5.7 x 10^{-8} mL/h); 3) a custom-designed and constructed recording platform that allows stable, convenient, and precise positioning of oocytes and the various electrodes. This stage also provides ample access to a stereomicroscope, a fiber light with dual goosenecks, and the four magnetic stands used to mount and position the current and voltage electrodes; and 4) fabrication of current and voltage electrodes from a type of thin-wall glass capillaries. These electrodes have the desired tip resistance (0.5–1.4 MΩ), can penetrate the oocyte cell membrane very easily, and cause minimum damage to oocytes.

Occasionally, the recording system does not work properly, as indicated by an unusually large or continuously increasing holding current, development of a white spot in the cell membrane around the current electrode, and unstable *Vm* traces in response to voltage commands. The possible causes are 1) a V_{DIFF} electrode system has a small air bubble or the tip of the V_{DIFF} electrode is not aimed properly toward the oocyte; 2) a voltage or current electrode has high resistance (*e. g.* >2 MΩ) or its holder is dirty from salt deposit; 3) the connection wire for a V_{DIFF} electrode is broken; 4) the D.C. gain is not set to IN during voltage clamp.

Here we have described a method for recording I_j from *Xenopus* oocytes. It allows a stable voltage clamp of two opposed oocytes. This method is easy to implement and appears to have no obvious limitations for analyzing the biophysical properties of GJs. However, we are not in a position to tell how it compares with other published methods. We hope that labs that are newly interested in setting up the double oocyte voltage-clamp technique will find this method worth considering.

ACKNOWLEDGMENTS:

We thank Haiying Zhan, Qian Ge for their involvement in the initial stage of technical development, Kiranmayi Vedantham for helping with the figures, and Dr. Camillo Peracchia for advice on the oocyte pairing chamber.

DISCLOSURES:

The authors have no conflict of interest.

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Figure 1

Click here to access/download;Figure;Figure 1 - Flattened.psd

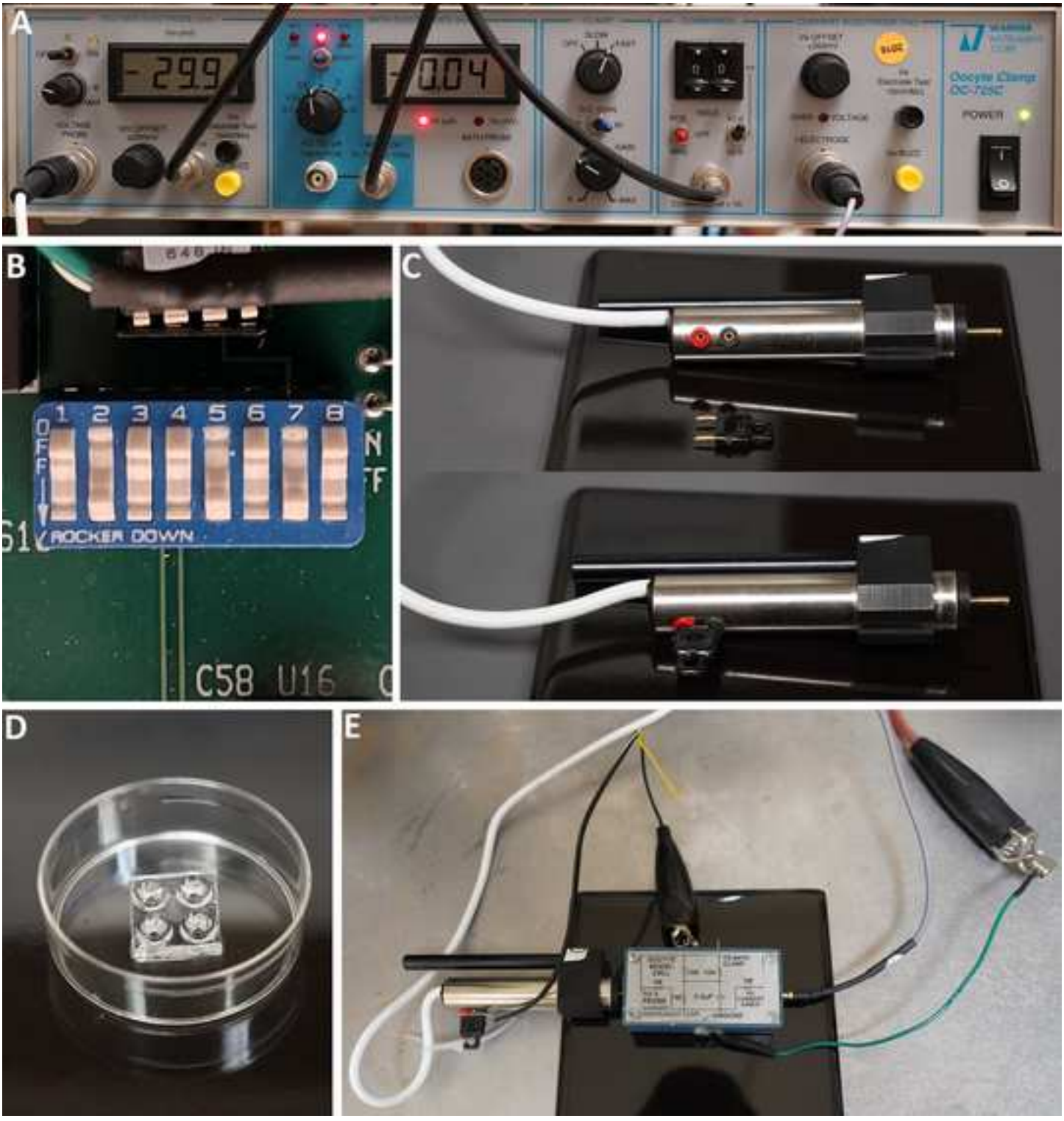

Figure 2

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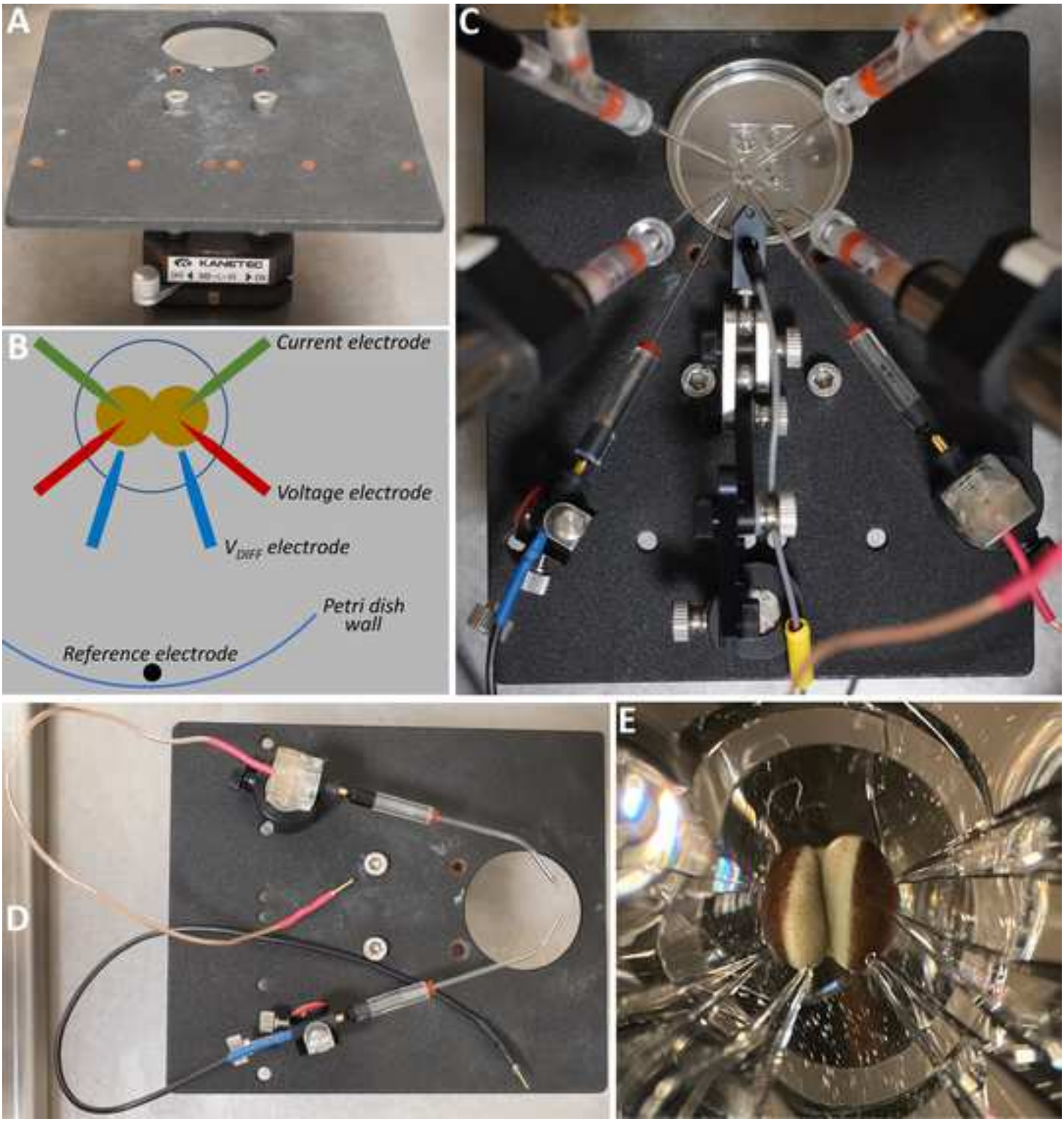
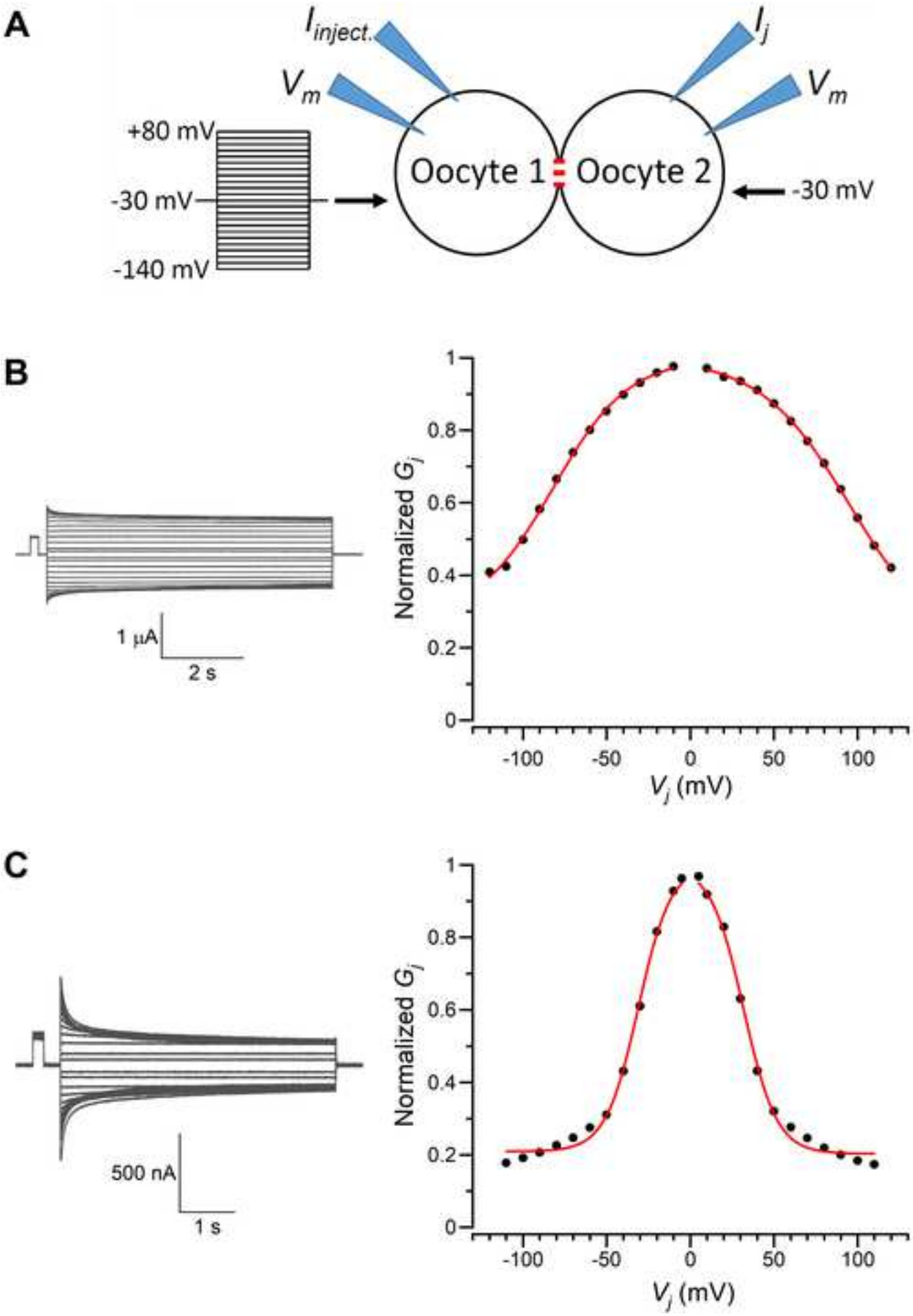


Click here to access/download;Figure;Figure 3-Final.tif



Click here to access/download;Table;Table 1 revised.xls

Table 1: Electrode Pulling Parameters

Step No.	Heat	Pull	Velocity	Time
1	260	…	40	200
2	240	…	40	200
3	240	60	40	200
4	245	100	60	200

Refer to the user manual at the manufacturer's website for definitions of the pulling parameters (https://www.sutter.com/MICROPIPETTE/p-97.html).

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Table of Materials
Table of Materials - Shui & Wang.xls

Rebuttal Letter

Click here to access/download;Rebuttal Letter;Response to reviewers 11-16-2021.docx

Dear editor,

We thank the reviewers for their careful review of the manuscript, and for their suggestions. We also thank you for the editorial comments. The manuscript has been revised accordingly. Below are our point-by-point responses to all the comments and suggestions:

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done.

2. Please provide at least 6 keywords or phrases.

Done.

3. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

Done.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We eliminated the personal pronouns wherever it was possible.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

Done.

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Done.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Done.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Fixed.

9. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Done.

10. Line 89-91: How long is the frog immersed in tricaine solution for anesthetization?

Described (line 102).

11. Line 93-94: Is there any specific volume of solution to be maintained for ovary immersion?

The manuscript now describes the diameter of the Petri dish (line 108). Neither the size of the Petri dish nor the volume of solution matters. We did not elaborate in the manuscript because it seems to be common sense.

12. Line 95: How is the incision closed? Suture (# size) or glue? Please specify the details.

The incision is closed by simple interrupted sutures using 5-0 silk suture, which is now described (line 109).

13. Line 98-99: Please mention how is the isolation confirmed?

It is now described as “isolated (solitary)” (line 109). The oocytes were initially connected. “Isolated” means that all the oocytes are separated. Please suggest a different word if the meaning still seems unclear to you.

14. Line 101: Any specific settings for stereomicroscope? Magnification?

We added “(≥ 25X highest magnification power)” after the word “stereomicroscope” (line 121).

15. Line 107-108: Large and healthy oocytes? How is this determined? Is there any specific size above which the oocytes are termed large and healthy?

We added this note to the manuscript: “large and healthy-looking oocytes” are those of stages V and VI showing no sign of over-digestion by the enzymes. (lines 132-133).

16. Line 118: How is the concentration measured using spectrophotometer? What is the absorbance?

The RNA synthesis kit that we use produces high-purity cRNA, as indicated by a high 260/280 nm absorbance ratio. However, the spectrophotometer that we routinely use, NanoDrop Lite, only reports the concentration. We apply 1 µl of diluted cRNA to the spectrophotometer to measure concentration. We added NanoDrop Lite to the Table of Materials. It seems unnecessary to describe how to use a NanoDrop in this paper.

17. Line 125-126: Please specify the centrifugation speed. Please express it as centrifugal force (x g) instead of revolutions per minute (rpm).

We added “(~16,000 RCF)” to the manuscript (line 150).

18. Line 126-127: Volume of the supernatant transferred?

We clarified by saying that “quickly transfer the entire supernatant”. (line 151)

19. Line 129-132: How is the injection carried out? Any specific settings required?

The volume of injection may be changed by adjusting toggle switches in the microinjector. It seems unnecessary to describe about this because 1) different labs may use different microinjectors, and 2) such information is contained in the user manual.

20. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Done.

21. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

Done.

22. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We paid attention to these points in revising the manuscript.

23. Is any ethical clearance required for performing this experiment? If so, please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

We added a note describing “The surgeries are performed following a protocol approved by our institutional animal care committee.” (lines 113-114)

24. Please move the Figure and Table Legends section to the end of the Representative Results section.

Done.

25. Please do not use the &-sign or the word “and” when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

Done.

26. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.

Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Done.

27. Figure 3: Please revise “sec” to “s” in panels B, C and D.

Done.

28. Please ensure that the Table of Materials contains all the supplies (chemicals, reagents, consumables, instruments, surgicals, etc.) used in this study.

Checked.

Reviewers' comments:
Reviewer #1:

Comment

Manuscript summary:
The dual whole cell voltage clamp technique was developed in order to evaluate properties of gap junctional conductance, and initially revealed that the endogenous connexin present in amphibian embryos is remarkably voltage sensitive (https://doi.org/10.1126/science.312530). At that time voltage clamps capable of passing the large currents necessary to clamp leaky oocyte membranes were not commercially available, but since then most labs studying biophysical properties of gap junction channels in oocytes and in mammalian cells have purchased off the shelf instrumentation. This methods submission claims that present usage of dual whole cell voltage clamp is "challenging" (l.27), and that modifications to the user manual for a specific product described herein are necessary to obtain stable recordings. Although the authors have certainly mastered the technique of dual whole cell recording as judged by their publications on C elegans innexins, there are concerns about novelty, accuracy of statements, and data presented.

Response

We thank the reviewer for pointing out this first paper on the dual oocyte voltage clamp technique published about 40 years ago. In the revised manuscript, we describe “The earliest successes of double voltage clamp of frog blastomeres were reported about 40 years ago[13,14]” (lines 72-73). In addition, we revised the sentence containing the word “challenging” to the following: “Because even the commercial amplifiers do not provide instructions for double oocyte voltage clamp, it is often challenging for new or less sophisticated electrophysiological labs to implement this technique.”(lines 77-79) This statement appears to be appropriate because it took us a long time to figure out how to perform this technique in spite of the fact that we had been doing other kinds of electrophysiological recordings for many years.

Comment

Major concerns:
My most serious concern is the I-V plot in Fig 3C, which is a rather linear representation of what should be a doubly rectifying relation. Not only does this misrepresent the

electrophysiological data, but the use of I-V relations is only justified if both cells' currents are displayed, and G-J curves must be shown to compare GJ properties.

Response

All the I_j-V_j plots in the original Figure 3 were based on instantaneous I_j. To address the reviewer's critique about this figure, we eliminated all the I_j-V_j plots, and used G_j based on steady-state I_j to plot the G_j-V_j relationships. The trace in Figure 3C was also replaced by a new one, which gives a slightly better appearance in the G_j-V_j curve. In addition, we eliminated Figure 3D because it is unnecessary for this publication.

Comment

A description of how such plots should be generated might be a helpful reference, although many such descriptions have appeared previously (e.g., DOI: 10.1038/nprot.2006.266).

Response

We thank the reviewer for this suggestion. In the revised manuscript, we describe the procedures that we use to generate G_j-V_j plots from the electrophysiological data. In addition, we direct readers to two publications for further information about the double voltage clamp technique, including the reference mentioned by the reviewer (lines 96-97).

Comment

The advance of this contribution appears to be minimally incremental as described: L.31: "Our major modifications [to manufacturer's instructions] include a custom-designed recording stage that allows convenient and precise positioning of paired oocytes and electrodes, use of bath solution as the conductor in voltage differential (VDIFF) electrodes, adaptation of a commercial low-leakage KCl electrode as the reference electrode, and use of magnetically based clamps to conveniently position the VDIFF and reference electrodes." As noted below, if sections on analysis and interpretation were amplified, value would be enhanced.

Response

The novelty of our method is limited to using a high side current measuring approach for dual oocyte voltage clamp. We feel that these modifications are very important to our success. The method described here is meant to be an alternative rather than a replacement for those used by others. As suggested by the reviewer, we added descriptions about our procedures for data analysis and interpretation.

Minor concerns:

COMMENT

L.44: Alternative splicing of gene transcripts may further increase the diversity of GJ proteins. While this may occur in innexins, it has not been reported in connexins.

RESPONSE

We have revised our description to "Alternative splicing of gene transcripts may further increase the diversity of GJ proteins at least for innexins[7,8]." (lines 48-49).

COMMENT
l.59. This overstates the situation with cell lines. Although HeLa cells are coupled, the most commonly used cell line for exogenous connexin expression is Neuro2A, which has virtually no endogenous GJ expression.

RESPONSE

We have revised our description to “Unfortunately, most commonly used cell lines except the Neuro-2A cell line are inappropriate for studying GJ biophysical properties due to complications by endogenous connexins.” (lines 62-64).

COMMENT
l.62. This understates the disadvantages of oocytes for such studies: Low input resistance, requiring high GJ expression and large currents and disallowing single channel recordings of GJ currents. The end of the paragraph criticizes the adaptation of two voltage clamps to study GJ currents, which is not justified.

RESPONSE

We agree with the reviewer that Xenopus oocytes have their own limitations for studying gap junction biophysical properties. Because this publication will mainly be in a video format to show how a technique is performed, it seems unnecessary to point out the limitations of using Xenopus oocytes. In the revised manuscript, the end of the paragraph has been modified to “The earliest successes of double voltage clamp of frog blastomeres were reported about 40 years ago[13,14]. Since then, many studies have used this technique to record I_j in paired *Xenopus* oocytes. However, essentially all the previous studies have been performed with either homemade amplifiers[12,15,16] or commercial amplifiers designed for recordings on single oocytes (GeneClamp 500, AxoClamp 2A, or AxoClamp 2B, Axon Instruments, Union City, CA)[8,17-20]. Because even the commercial amplifiers do not provide instructions for double oocyte voltage clamp, it is often challenging for new or less sophisticated electrophysiological labs to implement this technique.” We hope that the reviewer would find it appropriate.

COMMENT
l. 83. "Our failure with the OC-84 725C amplifier in the standard mode indicates that it is not easy to repeat others' successes in such recordings without detailed instructions. Here we describe our method in details so that other labs may easily implement this technique by following our procedures." Subsequent sections deal with preparation of oocytes and cRNA preparation and delivery, which could be very helpful for the novice. As could a description of data analysis.

RESPONSE

As suggested, the revised manuscript describes how we perform data analyses. We also direct readers to the reference suggested by the reviewer (DOI: 10.1038/nprot.2006.266) for additional information (lines 96-97).

COMMENT
l.182. It is imperative that long pulses be applied (in Fig 3, about 6 sec), because GJ current relaxation is slow. [On the other hand, because relaxation is slow, clamp speed need not be a priority, so that the shield between current and voltage electrodes is unnecessary].

RESPONSE

We thank the reviewer for this information, which we did not know before. To address the reviewer's comment, we have eliminated the words about the shield between current and voltage electrodes.

COMMENT
l. 243. Presumably MOhm, not mOhm

RESPONSE

We have corrected the typo.

COMMENT
l. 264. These currents (and the relevant conductances) are poorly described.

RESPONSE

In the revised manuscript, we expanded this paragraph to describe how the representative I_j traces were obtained, and how the homotypic gap junctions formed by UNC-9 and UNC-7b differ in I_j kinetics and G_j-V_j relationship. (lines 348-353)

COMMENT
l. 306 to end: Phrases "it would be nice", "bells and whistles", and question about how method compares to other labs do not seem appropriate here.
Figure 1 does not convey much information not in Figure 2.

RESPONSE

Those words in the last paragraph of the Discussion have been eliminated. We revised Figure 1 but did not delete it because some readers might find it helpful to understanding the protocol section.

Reviewer #2:

COMMENT

Manuscript Summary:
Pairing oocytes to characterize expressed gap junctions is a commonly used vehicle to study their biophysical properties. Shui and Wang described a detailed experimental protocol for using this technique with a commercially available oocyte amplifier. The protocol was very nicely organized, and many details were explained. I have no major issues, but just some minor suggestions.

RESPONE

We appreciate these comments of the reviewer.

Major Concerns:
none

Minor Concerns:
COMMENT

-line 135, … by touching 'something' under … could you be more specific or give one example of 'something'?

RESPONSE

The user manual of the microinjector has instructions on how to fabricate the injection needles, including a procedure of breaking a pulled tip to a larger size by breaking it with forceps. In the revised manuscript, we suggest readers to “prepare glass micropipettes for oocyte injections by following instructions in the user manual of the Auto-Nanoliter Injector.”

COMMENT

-line 145-146, hypertonic solution, could you provide a bit more info on hypertonic solution, such as percentage or osmolarity of the hypertonic vs regular solution?

RESPONE

The revised manuscript no longer mention the hypertonic solution because we do not use it.

COMMENT
-line 243, MΩ

RESPONSE

The typo has been corrected.

COMMENT
-line 290, low leakage KCl electrode, not quite clear to me by this line. Could you clarify a bit more on 'low leakage', concentration of KCl and resistance etc.?

RESPONSE

The reference electrode has a resistance of ~2.7 kΩ, and leaks electrolytes at a very slow rate (~5.7 x 10^{-8} ml/hr). We provides this information in the revised manuscript (lines 398-399). In addition, a web link to the specifications of the electrode is added to the Table of Materials.

COMMENT
-Fig. 1c, I am unable to tell the dipping switch positions even with the high resolution photo. May be a simple indication for each switch position (1-8 on or off for each?).

RESPONSE

In the revised manuscript, we describe in Figure 1 legend that “All the toggle switches except 2, 5, and 7 are in the OFF position to use the amplifier in the high side current measuring mode”.

COMMENT
-Fig. 3, Ij recordings showed a few transient maybe capacitance current lines at the Vj pulsing or Vm pulsing onset and offset. I believe that these are not part of Ijs and should not be in these recordings.

RESPONSE

We removed the capacitance currents in Figure 3 as suggested.