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Using fluorometry and ion-sensitive microelectrodes to study the functional expression of heterologously-expressed ion channels and transporters in *Xenopus* oocytes

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Abstract

The *Xenopus laevis* oocyte is a model system for the electrophysiological study of exogenous ion transporters. Three main reasons make the oocyte suitable for this purpose: (a) it has a large cell size (~1 mm diameter), (b) it has an established capacity to produce—from microinjected mRNAs or cRNAs—exogenous ion transporters with close-to-physiological post-translational modifications and actions, and (c) its membranes contain endogenous ion-transport activities which are usually smaller in magnitude than the activities of exogenously-expressed ion transporters. The expression of ion-transporters as green-fluorescent-protein fusions allows the fluorometric assay of transporter yield in living oocytes. Monitoring of transporter-mediated movement of ions such as Cl⁻, H⁺ (and hence base equivalents like OH⁻ and HCO₃⁻), K⁺, and Na⁺ is achieved by positioning the tips of ion-sensitive microelectrodes inside the oocyte and/or at the surface of the oocyte plasma membrane. The use of ion-sensitive electrodes is critical for studying net ion-movements mediated by electroneutral transporters. The combined use of fluorometry and electrophysiology expedites transporter study by allowing measurement of transporter yield prior to electrophysiological study and correlation of relative transporter yield with transport rates.

Keywords

ISE; EGFP; pGH19; Typhoon

1. INTRODUCTION

The capacity of *Xenopus laevis* oocytes^a to translate exogenous RNAs was first demonstrated nearly 40 years ago when Gurden and coworkers detected substantial expression of rabbit hemoglobin in frogs' oocytes that had been co-injected with hemin and a subfraction of rabbit

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^aThe oocytes of some other amphibians are also suited for exogenous protein expression studies: for example oocytes isolated from *Xenopus tropicalis* [83], the cane toad *Bufo marinus* [84,85], the fire-bellied newt *Cynops pyrrhogaster* [86], and the axolotl *Ambystoma mexicanum* [87].

reticulocyte RNA [1]. Early evidence that oocytes could be used to express exogenous ion transporters include demonstrations that oocytes injected with *Torpedo* electric organ mRNA [2,3] and those injected with denervated cat-muscle-mRNA [4] incorporate functional nicotinic acetylcholine-receptor-channels into their plasma membranes.

Once the cDNA encoding a transporter has been cloned, oocytes can be microinjected with the corresponding cRNA [5,6,7,8], allowing: (a) the study of that transporter in relative isolation—barring contributions from endogenous proteins [9]—forging a strong link between injected message and detected ion transport activity (e.g., ref. [8]) and (b) the efficient and rapid study of transporter mutants expressed from engineered cDNAs (e.g., [6]). The link between injected message and detected ion-transport activity has since been exploited by investigators aiming to elucidate the molecular identity of a particular ion-transporter activity. This process—expression cloning (reviewed in ref. [10])—can follow the production of soluble proteins or the manifestation of transporter activity in oocytes microinjected with increasingly sub-fractionated pools of mRNA until a single, causal mRNA species is isolated (for early examples see refs [11,12,13]). Oocytes are now routinely used for expressing and studying the function and regulation of wild-type and mutant ion transporters along with their associated protein-partners.

In this review, we describe a system in which we (a) microinject oocytes with cRNA that encodes a transporter fused to enhanced green fluorescent protein, (b) use fluorometry to assess the yield of the fusion protein in live oocytes, and (c) use ion-sensitive microelectrodes^b to assay the activity of expressed transporter fusion. The following two sections of the present review are written for the investigator who has ion-transporter cDNA in hand and who wishes to express that transporter in oocytes. Thus, section 2 brings together methods for producing cRNA, isolating oocytes and microinjecting cRNA into those oocytes and section 3 presents a series of experiments in which we assess the expression of an enhanced green fluorescence protein-tagged transporter in living oocytes using three different fluorometric methods. Section 4 of this review is written for the investigator who is already expressing ion transporters in oocytes and who wishes to study net transporter-mediated ion-movements using ion-sensitive microelectrodes (ISMs). Thus section 4 brings together methods for the fabrication, calibration, and utilization of intracellular and extracellular/surface ISMs. Section 5 of this review considers the utility of combining fluorometric and electrophysiological measurements.

2. EXPRESSING GREEN-FLUORESCENT-PROTEIN-TAGGED TRANSPORTERS IN *XENOPUS* OOCYTES

2.1 Introduction

Exogenous proteins can be produced in oocytes by injecting DNA into the oocyte nucleus [14] or by injecting—into the oocyte cytoplasm—either RNA [1,15] or DNA plus RNA polymerase [16,17]. In addition, it is possible to inject oocytes with exogenous membrane preparations [18,19,20,21], allowing the transfer of membrane proteins—in association with their native lipids—from human tissue samples into oocytes for electrophysiological study [22,23]. Arguably the most tractable method for studying wild-type and mutant transporters is the microinjection of transporter cRNAs. By this method, the quantity of injected transcript can be controlled and transporter activity may be detected in the oocyte plasma membrane in fewer than 24 hours [24] and monitored throughout the experimental lifetime of the oocyte^c. Because green fluorescent protein (GFP)^d [25] can be made to fluoresce when expressed in

^bMultiple other techniques can be used to assay the action of exogenous ion transporters in oocytes, including two-electrode-voltage-clamp (e.g., see methods in ref. [45]), patch-clamp [54], tracer-isotope influxes [10], trace-isotope effluxes (e.g., see methods in ref. [35]) and the use of ratiometric dyes (e.g., [88]).

living cells [26], the expression of proteins that are tagged with GFP [27]—including ion transporters [28]—can be tracked in live cells. In this section we bring together methods for producing oocyte-expression ready cRNA that encodes a transporter tagged with enhanced GFP (EGFP)^e, isolating expression-competent oocytes, microinjecting cRNA into oocytes, and using fluorescence to monitor the expression of the EGFP-tagged transporter.

2.2 Choosing plasmid vectors and preparing cRNAs for microinjection

Plasmids—The starting material for generating transporter cRNA is transporter cDNA that has been subcloned into a plasmid vector appropriate for *in-vitro* transcription [29]. Some commonly used—but non-commercially available—plasmids optimized for generating *Xenopus*-oocyte-expression-competent cRNAs include pGEMHE [30], its derivative pGH19 [31], pBSXG [32], and pTLN [33]. Typical features of a cRNA-transcription-competent plasmid, exemplified by pGH19, are shown in Figure 1. Three minimum requirements of such a plasmid are:

- A promoter sequence (e.g. T7, SP6) upstream of the transporter cDNA open reading frame (ORF) to drive RNA transcription.
- A multiple cloning site to allow subcloning of the transporter cDNA downstream of the promoter.
- A unique restriction site downstream of the transporter ORF that allows vector linearization and ensures efficient transcriptional termination.

The commercially available TOPO TA vector (Invitrogen, Carlsbad, CA) meets both criteria, and has been used successfully to produce oocyte-expression competent cRNA [34,35]. Four optional features can be incorporated into a cRNA-transcription-competent plasmid construct:

- cDNA encoding the 5'- and 3'-UTRs of *Xenopus* β -globin (pink boxes in Figure 1) to flank the transporter ORF and thereby enhance—sometimes markedly [36]—translational efficiency of the message [30].
- Placement of the initiator ATG codon of the transporter ORF into the context of a Kozak sequence also to enhance translational efficiency of the message (i.e., GCCACCATG in which the initiator ATG codon of the transporter is underlined [37]).
- A polyadenosine tract (yellow box in Figure 1) which is transcribed as a poly(A) tail—downstream of the transporter ORF to lengthen the useful lifespan of the cRNA post-injection [38,39].
- A polycytosine tract (orange box in Figure 1) downstream of the polyadenine tract to protect the poly(A)-tail from exonuclease activity [40,41].

cRNAs also can be prepared from purified PCR products as long as the product includes a suitable promoter sequence [42]. This approach is especially useful for generating cRNA

^cOocytes may survive in culture for more than 7 days, but we typically inject fresh oocytes on a weekly basis, discarding the old ones. The optimal post-injection incubation period for transporter expression and experimentation will vary depending on the transporter being expressed and is determined by the individual investigator.

^dModification to the GFP structure produces variants with different fluorescence excitation/emission spectra, such as red fluorescent-protein (e.g., DsRed), yellow fluorescent-protein (YFP), and cyan fluorescent-protein (CFP). These variants are particularly useful for co-expression studies or in protocols where the cells are injected with a ratiometric dye (e.g. BCECF) whose fluorescence signal interferes with that of GFP. Additional functionality can be gained by using variant GFPs that are, for example, pH-sensitive, or Ca⁺⁺-sensitive (reviewed in ref. [89]).

^eEGFP is a mutant GFP that has a 35-fold enhanced fluorescence intensity, when excited at 488 nm, compared to wild-type GFP [90, 91] and a codon usage optimized for expression in mammalian cells [92].

species that correspond to fragments of a cDNA without the need to subclone each fragment separately.

Considerations for the EGFP-tagging of transporters—The creation of a plasmid construct that encodes a transporter as an EGFP fusion requires that cDNA encoding the EGFP open-reading-frame (amplified by PCR from the pEGFP-C1 template; Clontech, Palo Alto, CA) be subcloned in-frame with the transporter's open-reading-frame, such that the two proteins are translated as a single chimeric polypeptide. GFP is a 238-aa protein, the structure of which has been described as a 'β-can'; a barrel composed of 11 antiparallel β-strands surrounding a fluorophore-containing α-helix [43,44]. Structural models of GFP represent both the extreme amino- and carboxy-termini (Nt and Ct) as unstructured protrusions projecting from the top of the 'can' [43,44], such that both termini are available for fusion to a transporter without disruption of the fluorophore. Indeed, EGFP has been used to tag both the Nt and Ct of membrane proteins. For example, the electrogenic sodium bicarbonate transporter NBCe1—whose Nt and Ct are cytoplasmic—can be tagged at either terminus without disrupting transporter function [45,46]. A caveat to this example is that when EGFP was fused directly to the Nt of NBCe1, the chimera was inactive; restoration of function required the introduction of a 20-aa linker between the GFP and the Nt of NBCe1 (Boron lab, unpublished data). GFP can also be used as an extracellular tag [47,48] for membrane proteins whose Nt or Ct are extracellular. A caveat to adding an extracellular-Nt tag is that the EGFP ORF has no intrinsic signal sequence and therefore must be inserted downstream of the transporter's own signal sequence to ensure that the EGFP is secreted^f (e.g., ref. [47]).

The primary consideration, once a transporter/EGFP chimera has been constructed, is that the fusion protein retains the properties of the untagged transporter, which in some cases it may not (e.g., [49,50,51]). In one study of the ionotropic glutamate receptor (GluR3) expressed in *Xenopus* oocytes, the receptor was tagged with GFP either at its extracellular Nt (Nt-GFP-GluR3) or cytoplasmic Ct (GluR3-GFP-Ct) and the molecular function of the chimeras was compared to that of the untagged receptor [47]. GluR3-GFP-Ct expression conferred, to the oocyte plasma membrane, agonist-evoked conductances of greater magnitude than those conferred by either untagged GluR3 or Nt-GFP-GluR3 because oocytes expressed GluR3-GFP-Ct to the plasma membrane more efficiently than either of the other two constructs. However, GluR3-GFP-Ct exhibited a substantially lower affinity for two agonists than the untagged receptor. Moreover, certain agonist-evoked currents mediated by GluR3-GFP-Ct deactivated more slowly. On the other hand, the molecular function of the Nt-GFP-GluR3 chimera accurately reflected the behavior of the untagged receptor for all the criteria tested. A caveat is that even the abnormal GluR3-GFP-Ct behaved normally with respect to its sensitivity to a third agonist as well as its sensitivity to two antagonists. Thus, one must be careful when judging the appropriateness of a GFP-tagged construct.

Preparation of capped cRNAs—Critical for producing high yields of good quality RNA is the maintenance of an RNase-free environment. The extent to which precautionary measures are necessary varies from lab to lab, but include: (a) wearing gloves, lab coats, face masks, and even caps during RNA manipulations, (b) using an RNase removal solution such as RNaseZap (Ambion, Austin, TX) to prepare surfaces and equipment, (c) setting aside dedicated pipettes, pipette tips, and reagents specifically for use during RNA preparation, and (d) abstaining from

^fInterestingly a BK channel GFP-tagged at its extracellular Nt seemingly without an added signal sequence is appropriately exported in HEK293 cells [93] and CHO cells can secrete GFP with no signal sequence—albeit in a misfolded and non-fluorescent confirmation into—the extracellular medium [94].

the use of RNase A during plasmid DNA preparation[§]. Figure 2 provides a workflow for preparing purified, capped cRNA from a cDNA template.

2.3 Preparing defolliculated oocytes

The maintenance of *Xenopus* females and the harvesting of ovaries is covered in detail elsewhere in this volume [52]. Animal care and surgery protocols should be devised in concert with the animal care facility at the investigator's institution. This section concerns the preparation of oocytes from surgically pre-extracted ovaries obtained in-house or by mail order (Nasco, Fort Atkinson, WI). The composition of solutions used during oocyte isolation and culture is provided in Table 1. A healthy *Xenopus laevis* ovary contains in excess of 1000 experimentally useful oocytes. Each ovary consists of a lobed, vascular sac of connective tissue, within which each oocyte is surrounded by a vascularized layer of follicle cells (see below). Individual oocytes can be removed manually from the ovary, although in practice it is expedient to liberate all the oocytes at once by enzymatic digestion of the connective tissue. Enzymatic treatment also removes the follicular layer, which is recommended because follicle cells: (a) represent a physical barrier to impalement of the oocytes with the microinjector needle and microelectrodes, and (b) have their own endogenous transporters and are coupled to the oocytes cytoplasm via gap junctions [53], complicating the measurement and interpretation of physiological data. The removal of the follicular layer reveals the so-called vitelline membrane that surrounds the oocyte plasma membrane. Removal of this connective-tissue 'membrane' is necessary for patch-clamping studies [54] but is not desirable for the protocols described in this review as the vitelline membrane helps to maintain oocyte integrity. The protocol in Figure 3 describes an enzymatic digestion that liberates individual oocytes from the ovary and from their follicular layers, leaving the vitelline membrane intact.

2.4 Sorting, microinjecting, and culturing oocytes

Sorting—The ovary digestion protocol described in Figure 3 results in the isolation of a mixed population of single oocytes. Examples of experimentally useful oocytes are shown in Figure 4a. Useful oocytes are large (> 1 mm diameter), spherical, and have a clear color separation or an unpigmented band between their light (vegetal) and dark (animal) poles. Morphologically these oocytes are categorized as Dumont stages IV–VI [55], which are those demonstrated to have the highest translational capacity [56,57]. Useful oocytes must be separated—or sorted—from non-useful oocytes as soon as possible after isolation, and transferred into sterile OR3 medium. Non-useful oocytes (Figure 4b) are those that are immature (small), discolored, shriveled, over-digested, unusually shaped, damaged, retain their follicular layer (Figure 4b, inset), or remain attached to connective tissue.

Sorting of useful oocytes is performed under a dissecting microscope using a sterile glass Pasteur pipette^h. Sorted oocytes may be injected on the same day as their isolation, but it is preferable to leave the cells overnight at 18°C prior to injection to allow identification and removal of those few percent of apparently-healthy but non-robust oocytes that exhibit signs of poor health within 24 hours of isolation.

Microinjecting—cRNA can be injected into oocytes using a Nanoject II Variable Volume Automatic Injector (Drummond Scientific Company, Broomall, PA). Injection needles are pulled from 10-μl microdispenser capillary glass (Cat.No. 3-000-210-G, Drummond), using, for example, a Model P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Company,

[§]Detailed technical notes about creating an RNase-free environment can be found online at <http://www.ambion.com/techlib/basics/rnasecontrol/index.html>

^hTo avoid mechanical stress on the oocytes during handling, the tip of the glass pipette should be broken to a diameter of ~5 mm and flame polished.

Novato, CA). The capillary glass is drawn on the puller to produce two ‘needles’ with tips that are effectively sealed. The sealed ends are then manually broken (under a dissecting microscope using either a sterile razor-blade or fine-tipped forceps) to give a ~50- μ m-diameter tip. The exact diameter of the tip is not important but if the tip is too narrow the needle will clog easily, and if the tip is too wide impalement will damage the oocyte. Needles are backfilled with mineral oil, attached to the microinjector and the solution of RNA in H₂O is drawn into the tip of the needle. Oocytes are injected in a sterile Petri dish that is filled with OR3 medium, the bottom of the dish having been scratched with a sterile razor blade to create a rough surface that keeps oocytes in place during impalement.

The amount of cRNA that needs to be injected per oocyte to provide a useful level of transporter expression should be determined for each cRNA species. The injection quantity can be controlled by diluting the cRNA or by injecting more or less volume of cRNA: oocytes can easily tolerate a 46 nl injection pulse. A control group of oocytes should be injected with the equivalent volume of sterile H₂O. Following injection, damaged oocytes should be removed and the remaining oocytes transferred to sterile OR3 medium.

Culturing—Injected oocytes are maintained in sterile OR3 at 18°C. For convenience, subpopulations of injected oocytes can be kept in separate wells of a 6-well tissue culture dish, with no more than 50 oocytes per well. Oocytes should be transferred to fresh media daily. Oocytes showing signs of discoloration or ill-health should be discarded.

3. IMAGING EGFP-TAGGED TRANSPORTERS IN INTACT (LIVING) OOCYTES

3.1 Visualizing fluorescence with a confocal microscope

EGFP fluorescence can be visualized in living oocytes using a confocal microscope such as a FluoView™ FV1000 (Olympus, Center Valley, PA), equipped to allow 488-nm excitation and 510-nm band-pass–emission detection. Confocal images can be analyzed to provide a relative quantification of expression [58] and has even been used to follow the recycling of an EGFP-tagged transporter from the oocyte plasma membrane into endosomal-like structures below the membrane surface [59]. Living oocytes can be visualized under a confocal microscope, in 500 μ l of ND96 solution contained in a Lab-Tek™ 8-chambered coverglass (Cat.No.#155411, Thermo Fisher Scientific Inc., Waltham, MA)ⁱ. Figure 5 shows confocal images of H₂O-injected oocytes, oocytes expressing the human sodium bicarbonate cotransporter NBCe1-A [60] fused at its Ct to EGFP (NBCe1-EGFP-Ct), and oocytes expressing soluble EGFP (sEGFP). In a focal plane (FP) near the base of the oocyte (red line FP₁ in Figure 5a), a H₂O-injected oocyte displays no intrinsic fluorescence (Figure 5b and c). However, a cell expressing NBCe1-EGFP-Ct exhibits a fluorescence that appears, except for macroscopic folds in the membrane, to be evenly distributed throughout the visualized patch of membrane (Figure 5d; similar examples for other GFP-tagged transporters are shown in refs. [48,61]). This pattern is similar to the distribution of fluorescence—in the same focal plane—in a cell expressing sEGFP (Figure 5e). This is to be expected, as the laser is able to penetrate the cell to excite EGFP molecules in the cytoplasm close to the coverslip. Thus, it is likely that in Figure 5d, not all of the fluorescence originates from NBCe1-EGFP-Ct that is in the plasma membrane. Some may represent NBCe1-EGFP-Ct in the membrane of vesicles beneath the plasma membrane.

In a focal plane nearer the equator of the oocyte (red line FP₂ in Figure 5f), a H₂O-injected oocyte displays no intrinsic fluorescence (Figure 5g and h). However, a cell expressing NBCe1-EGFP-Ct exhibits a fluorescence that appears as a ring at the oocyte perimeter (Figure 5i;

ⁱCells can also be visualized within a droplet of ND96 that is maintained on a coverglass by a hydrophobic circle drawn with a PAP pen.

similar examples for other GFP-tagged transporters are shown in refs. [48,61]). In some cases, transporter expression is polarized to favor either the light vegetal or the darker animal pole of the oocyte [48,47]. Interpretation of fluorescence images such as that in Figure 5i can be confounded in two ways. Firstly, as mentioned above, the laser could excite NBCe1-EGFP-Ct molecules in vesicles close to the plasma membrane. Indeed, even the fluorescence of soluble EGFP appears to be confined to the cell surface in intact oocytes visualized in this manner (Figure 5j). Secondly, because the laser cannot penetrate deep into the cell, any NBCe1-EGFP-Ct molecules that happen to be located deep beneath the plasma membrane cannot be visualized in Figure 5i. Indeed, it would appear from Figure 5j that sEGFP is absent from the deep cytosol, even though we might suspect from that it is present in this compartment (see below). Thus a peripheral distribution should not be taken as proof of exclusive plasma-membrane localization of a transporter without further qualification (e.g., by biotinylation or functional assay, for example see ref. [62]).

In order to visualize transporters that are expressed in an internal compartment of the oocyte, it is necessary to study fixed and embedded oocyte sections (for example, see refs. [63] and [45]). A caveat is that the GFP fluorophore may be destroyed during sample preparation. However, under these circumstances, EGFP-tagged transporter can be immunodetected with an anti-GFP antibody [45]. In unpublished work, we have immunodetected sEGFP throughout fixed and embedded equatorial sections of oocytes.

3.2 Measuring fluorescence with a plate reader

The fluorescence of oocytes expressing an EGFP-tagged transporter can be quantified on a plate reader such as the POLARstar Fluorescence Polarization Microplate Reader with FLUOStar software (serial no. 403–0513, BMG Labtechnologies, Durham, NC). Each oocyte is transferred into a single well of a black polystyrene 96-well plate with a clear bottom (Product #3601; Corning Inc., Lowell, MA). The plate reader is set to fluorescence-intensity mode, and the fluorescence of each oocyte is measured with a 485-nm excitation filter and a 510-nm band-pass–emission filter. Fluorescent signals from EGFP-expressing oocytes are typically only 10% greater than the ‘background’ readings obtained from H₂O-injected oocytes or a well containing only ND96 solution. Thus, multiple measurements must be taken per oocyte to limit error. In the example presented in Table 2/column 2, the fluorescence from each well was averaged from 3 replicate readings each consisting of 200 excitation flashes. At the level of individual oocytes the fluorescence of NBCe1-EGFP-Ct expressing cells correlates well with detected transporter activity; the Pearson correlation coefficient was 0.8 for a similar set of oocytes [45]. A disadvantage to this approach is that manipulation of the oocytes into and out of the wells—which are non-sterile—risks cell damage and the possibility of contaminating the oocyte culture.

Table 2/column 2 shows averaged data from an experiment in which the fluorescence of groups of 12 oocytes was measured using a plate reader. Oocyte groups were injected with either cRNA encoding NBCe1-EGFP-Ct, cRNA encoding sEGFP, or H₂O 4 days prior to assay. The groups expressing either NBCe1-EGFP-Ct or sEGFP exhibit a substantially greater fluorescence than the H₂O-injected group.

3.3 Measuring and visualizing fluorescence with a variable mode imager

A Typhoon Trio+ Variable Mode Imager in concert with the colony-counting function of the ImageQuant TL software package (GE Healthcare, Piscataway, NJ), can be used to quantify the fluorescence of oocytes bathed in OR3 medium, in the sterile environment of their culture dish. The fluorescent image is obtained using a 485-nm excitation filter and a 520-nm bandpass emission filter. Some manipulation of the cells may be necessary to ensure oocytes are distanced from each other to allow accurate measurements. Figure 6a-d show fluorescence

images of oocytes, still in their culture dish, expressing either NBCe1-EGFP-Ct, sEGFP, or no EGFP (i.e., H₂O-injected oocytes). The average fluorescence intensity of each group of cells is presented in Table 2/column 3. The groups expressing either NBCe1-EGFP-Ct or sEGFP exhibit a substantially greater fluorescence than the H₂O-injected group. To track individual oocytes it may be necessary to image the cells in individual wells of a 96-well plate. The cells imaged in a 96-well plate in Figure 6e are the same oocytes whose fluorescence was assayed by plate-reader (see previous section and Table 2/column2). The fluorescence intensity of individual oocytes measured using the imager and the plate reader correlate well (Pearson correlation coefficient is 0.66).

4. FABRICATION AND USE OF ION-SENSITIVE MICROELECTRODES

4.1 Introduction

The type of ion-sensitive microelectrodes (ISMs) discussed in this section are single-barreled and employ ion-sensitive cocktails—first used by Ross in 1967 [64] and reviewed in refs. [65,66]—as opposed to those microelectrodes that are fabricated from ion-sensitive glass (e.g., see ref. [67]). ISMs filled with a liquid cocktail are quick to manufacture in large numbers, can be easily modified for a wide variety of analytes (e.g., Cl[−], H⁺, K⁺, Na⁺), and are relatively durable—in oocyte experiments, a good electrode should be usable for many weeks. ISMs respond to changes in ion activity, not ion concentration^J. For a full consideration of the theory behind the use of ion-sensitive electrodes, see ref. [68]. In this section we consider the use of two types of ISM: (1) ‘sharp’, intracellular ISMs—to measure intracellular ion-activity—that are introduced into an oocyte and (2) ‘blunt’, surface ISMs—to measure changes in ion activity in the vicinity of the surface of the oocyte—that are pushed against the oocyte surface to create an enclosed, extracellular measurement-site (an enhancement of the natural “unstirred layer” that surrounds the plasma membrane).

4.2 Fabricating ion-sensitive microelectrodes

Preparing capillary glass—The measurement of intracellular ion-activity requires two microelectrodes: an ISM containing an ion-selective cocktail and a ‘sharp’, KCl-filled microelectrode for use as a reference. The same glass capillaries are used in the fabrication of both microelectrodes. That is, one uses a thin-walled borosilicate glass capillary tubing that contains a filament (e.g., OD = 2 mm, ID = 1.56 mm, length = 10 cm; Model No. GC200TF-10; Warner Instruments LLC, Hamden, CT) and pulls each piece into two electrodes with tapered points using a micropipette puller (e.g., Model P-97 Flaming Brown, Sutter). For oocyte experiments, the reference electrode tip resistance, when filled with saturated KCl (Cat.No.# SP138-500; Thermo Fisher Scientific Inc.), should be 0.5–1.5 MΩ. This KCl-filled microelectrode requires no further modification for use as a reference for an ISM and is also suitable for measuring oocyte membrane potential (V_m).

ISMs require several extra manufacturing steps. The pulled glass capillaries that—is, empty microelectrodes (specimens that have never been filled with KCl)—are baked overnight in an oven at 200 °C to remove moisture. The—next and trickiest—step is to apply a hydrophobic coating to the glass by vaporizing silane onto its surface. Silanization, as this process is known, is performed with the unfilled microelectrodes still in the oven. The electrodes are held—tip up—in an open metal rack (the cylindrical holes in which the electrodes sit allow access to the

^JThe activity of an ion in solution is not the same as its concentration. Activity is the effective concentration of an ion in solution, representing its chemical ‘availability’. Activity is defined by the chemical potential of an ion in solution compared to its activity under standard conditions and is influenced by factors such as temperature, pressure, and interactions with other ions in the solution. At low concentrations of the ions considered here (< 10 mM), the concentration may be an overestimate of the activity by ~10%, rising to ~20% at concentrations of 100 mM [68]. For most of the measurements considered in this review, the disparity is small enough to be ignored. In this review, we use the term ‘concentration’ when the activity of the ion is unknown.

interior of the capillary glass from beneath) that rests on a Pyrex[®] petri-dish. An inverted glass jar placed onto the petri-dish creates a sealed environment around the electrodes/rack. The jar is briefly lifted to allow the injection of 80 μ l of bis(dimethylamino)dimethylsilane (Cat. No. 14755; Fluka Chemical, Milwaukee, WI) onto the petri-dish, below the rack. Finally, the jar is replaced so that the capillaries are exposed to an atmosphere of silane vapor. The silane coating provides an unbroken hydrophobic surface to which the ion-sensitive cocktail can adhere, thereby: (a) spatially stabilizing the cocktail at the tip of the microelectrode and (b) eliminating aqueous pathways between the cocktail and inner surface of the microelectrode that would otherwise provide a route by which ions (i.e., electrical current) could bypass the cocktail. After 10–15 minutes the jar is removed to allow the silane coating to cure. If the electrodes are over exposed to silane, there is a chance that the electrode tip will become clogged. Silanized capillary glass can be stored indefinitely in the oven until ready to fill with ion-sensitive cocktail. A more detailed consideration of silanization is provided in ref. [69].

Surface ISMs are prepared in a different manner to the ‘sharp’ intracellular ISMs discussed above because they require a wide, blunt tip. Such electrodes are pulled from standard-wall borosilicate tubing (e.g. OD = 2 mm, ID = 1.16 mm, length = 10 cm; Model No. GC200F-4; Warner Instruments) which has a smaller ID than the glass used to fabricate intracellular ISMs. Prior to silanization, surface ISMs are given a blunt tip (5–15 μ m) by breaking off and fire-polishing the pipette with a microforge as one would for a giant-patch pipette [70].

Filling and back-filling ISMs—A number of ion-sensitive cocktails with different ion-sensitive properties can be purchased from Sigma-Aldrich (Table 3). The cocktails are introduced into the electrode tip using a syringe attached to either a thin fused silica tubing (e.g., MicroFil MF34G, World Precision Instruments Inc., Sarasota, FL) or a glass capillary pulled to long, tapered point—either by hand over a Bunsen-burner flame or using a vertical micropipette-puller. Only a very small amount of cocktail needs to be added to the tip of each electrode— the use of too much cocktail will result in a high tip resistance decreasing the speed of electrode response. For an intracellular ISM, we typically create a 2-mm column of cocktail. A larger column of ion-selective cocktail (~ 4 mm) is incorporated into surface ISMs due to their wider tip. Despite the wider tip, the column cocktail should not leak out from a surface ISM. The ability of the surface ISM tip to retain the column of cocktail throughout an experiment depends on a number of subtle factors such as the precise shape of the electrode, the geometry of the tip, and the effectiveness of the silanization process.

Any bubbles within the cocktail in the electrode tip should be coaxed out using a thin filament, such as a cat’s whisker. By applying pressure, one can force some of the cocktail out of the tip of the electrode, which has the advantage of reducing electrode resistance, which in turn reduces noise while increasing the speed of response. The electrical contact between the cocktail and the microelectrode circuitry is mediated by a ‘backfill’ solution that extends from the cocktail to the tip of the silver wire of the microelectrode holder. The recipes for the backfill solutions used in conjunction with certain ion-sensitive cocktails are presented in the third column of Table 3. The backfill solution is introduced into the microelectrode— injected under the meniscus of the cocktail to avoid an air gap at the cocktail/backfill interface—using a syringe attached to MicroFil MF28G tubing (World Precision Instruments Inc.). An alternative method for filling the tip of ISMs is to leave an air-gap between the electrode tip and the cocktail, to backfill, and then to apply positive pressure behind the backfill solution to force the air out of the tip of the electrode [71].

Storing ISMs—Filled and back-filled ISMs are stored tip-down, affixed via modeling clay to the inside wall of a light-proof jar, such that the tips of the electrodes are submerged beneath the surface of a suitable electrode storage solution. The composition of the storage solution should approximate the composition of the oocyte media in which measurements will be made

for example we store pH microelectrodes—made with H⁺-sensitive cocktail—in Buffer Solution pH 6.00 (Cat.No. #SB104-1; Thermo Fisher Scientific). ISMs can be stored at room temperature in this way for many weeks. However, the buffer solution can grow mold if not replaced regularly.

4.3 Using Ion-sensitive microelectrodes

Perfusion system for electrophysiological measurements—The sample, usually an oocyte bathed in a close-to-physiological solution such as ND96, is placed within a polycarbonate oocyte recording chamber (Warner Instruments LLC) and perfused with a solution (hereafter referred to as the ‘chamber solution’) that is delivered to the chamber via silicone or Tygon[®] tubing^k from a plastic syringe. Solution flow to one end of the chamber is maintained at 3–4 ml/min using infusion syringe pumps (Harvard Apparatus, South Natick, MA) or using a controlled gravity-flow perfusion system. Laminar flow of solution through the chamber is achieved by controlled aspiration of the perfusate from the opposite end of the chamber. If the oocyte is to be exposed to more than one solution, changeover between solutions can be achieved using pneumatically operated valves (Clippard Instrument Laboratory, Cincinnati, OH) or a manually operated multi-channel perfusion valve control system (Warner Instruments LLC). Experiments are typically performed at room temperature (~22°C). The perfusion system is flushed with distilled H₂O daily, and 70% ethanol weekly to prevent the build up of salts and contaminants.

Basic electronic configuration for ISM data acquisition—In order to minimize noise interference, the recording chamber is mounted on a pneumatic vibration-isolation table (Newport Corporation, Irvine, CA) within a Faraday cage and all components within the cage are individually connected in parallel to a single common electrical ground. Keeping the set-up clean is also critical to noise reduction. The basic electrical configuration for acquiring data from an ISM is represented in Figure 7a. An immersed silver or platinum wire (we prefer platinum as it is more inert and therefore less potentially cytotoxic) provides the electrical contact between the chamber solution and electrical ground. Electrodes are housed in microelectrode holders such as ESW-F20V (Warner Instruments LLC). Chloridized (i.e., anodized in saturated KCl or immersed in bleach for 10–15 min) silver wires built into each holder provide the electrical contact between the electrode backfill and the electrometer headstage. Ion-selective measurements are acquired using a high-impedance dual-channel differential electrometer (e.g., Model FD223, World Precision Instruments Inc., Sarasota, FL). Channel A of the dual-channel electrometer acquires data from a headstage connected to an ISM while Channel B of the electrometer acquires data from a headstage connected to a KCl-filled reference electrode (see 4.2 Fabricating ion-sensitive microelectrodes). The tips of both microelectrodes are positioned as close as possible to each other at the sample-measurement site using micromanipulators (World Precision Instruments Inc.), a feat more easily achieved if the oocyte recording chamber is viewed through a dissecting microscope. The difference between the outputs from channel A and channel B (channel A – channel B) is proportional to the ion activity of the sample and is independent of variations in electrochemical potential between samples. Data are acquired via a digitizer (e.g. Digidata from Warner Instruments LLC) connected to a computer. Data are visualized and recorded using software such as the Axoscope module of pClamp (Molecular Devices, Sunnydale, CA).

Calibration of ISMs—Prior to electrophysiological recording, it is necessary to calibrate the ISM. This process enables the investigator to (1) determine whether the ISM responds appropriately to controlled changes in ionic concentrations and thus whether it is useful and (2) convert the differential output from the electrometer into an approximate measure of ion

^kTo deliver solutions that contain CO₂/HCO₃[–] one should use Tygon[®] tubing that, unlike the silicon tubing, has as low gas permeability.

concentration^l that can be monitored during the experiment. Calibration of the ISM is performed in the recording chamber. The aim is to obtain a linear plot of the differential output vs the log of ion concentration for a series of standard solutions. The composition of the calibration solutions should be close to that of the experimental solutions—or oocyte cytoplasm—except for variations in the concentration of the ion of interest. The variations in ion concentration in the standard solution ought to span two log scale decades and encompass the concentrations of ions to which the ISM will be exposed during the experiment. In accordance with the Nernst equation, the ideal potential change for a ten-fold increase in monovalent ion activity should be of 58.5 mV at room temperature. Although calibration slopes of 58.5 mV/decade are not always achieved, an electrode response that is greater in magnitude than 50 mV/decade is acceptable. There are three important points that must be considered:

1. Each ISM has a lower-limit of detection, usually in the 1–5 mM range, and a higher-limit of detection beyond either of which its response to changes in ion concentration is not linear. This is partly due the increased disparity between ion activity and ion concentration at higher ion concentrations^l.
2. No ion-sensitive cocktail is perfectly selective toward its target ion. Sensitivity to non-target or ‘interfering’ ions can cause the calibration slope to deviate from the ideal, and this deviation is especially pronounced at low concentrations of the ion of interest. In the case where the calibration slope of an ISM is < 50mV/decade due to non-target-ion interference, ISMs should be calibrated in—and the experiments performed using—solutions that contain as low a concentration of the interfering ion as possible. For example, some K⁺-sensitive cocktails are partly Na⁺-sensitive and must be calibrated in Na⁺-lacking solutions^l. However, when used in an intracellular ISM, the K⁺-sensitive cocktail is only exposed to the high-K⁺, low-Na⁺ environment of the oocyte cytoplasm—and not exposed to the low-K⁺, high-Na⁺ environment of the extracellular perfusate—enabling the use of close-to-physiological perfusion solutions during experiments. For similar reasons the interference of divalent cations with some Na⁺-sensitive cocktails is not an issue once the ISM has been calibrated and its tip introduced into an oocyte.

In some experimental situations, an interfering ion may be introduced to the recording chamber by a solution change, causing a shift in the ISM responsiveness in mid-experiment. In this situation the ISM should be calibrated twice prior to the experiment—once in the absence of the interfering ion and once in its presence. The appropriate calibration can be applied to data from the corresponding experimental period once the experiment is finished (e.g., see ref. [35]). Ideally the ISM should be similarly and usefully responsive under both sets of conditions (i.e., the magnitude of both calibration slopes are > 50 mV/decade).

Intracellular ISM recordings—The electronic configuration shown in Figure 7a is sufficient to measure intracellular ion-activity if both the intracellular ISM (that responds to the activity of a specific ion plus electrochemical potential) and its KCl-filled reference microelectrode (that responds only to electrochemical potential) are introduced into the oocyte. Again, the intracellular ion-activity is proportional to the difference between the outputs of the ISM and its reference electrode. A different configuration, such as that shown in Figure 7b, must be used if the simultaneously measurement of oocyte membrane potential (V_m) is desired. The optional monitoring of V_m throughout the experiment is useful both as a means of visualizing charge movements that might accompany ion transport activity, and as a proxy for oocyte ‘health’. V_m is described by the output differential between the ISM-reference electrode

^lWhen measuring [K⁺] or [Cl[−]] using an ion sensitive electrode it is important to remember that the reference electrode is filled with KCl, a slow leak of which may contaminate the measurement site. One possibility is to use an alternative solution to fill the reference electrode. For example, 0.24 M NaCl and 0.76 M Na-Acetate [95].

(hereafter, and in Figure 7b, referred to as the ' V_m ' electrode) and the electrochemical potential of the chamber solution (monitored by a low-resistance, KCl-filled microelectrode hereafter, and in Figure 7b, referred to as the chamber electrode ' V_{ch} '). If the potential of the chamber solution is held at 0 mV using bath-clamp circuitry, oocyte V_m is directly reported by the output from the V_m electrode (technically, $V_m - V_{ch}$, where $V_{ch} = 0$ mV). In the configuration shown in Figure 7b, the bath potential is held at 0 mV using a bath-clamp headstage of an oocyte clamp such as the OC-725C (Warner Instruments LLC). The I_{SENSE} port of the headstage is connected to V_{ch} (the tip of which is immersed in the chamber solution close to the oocyte). The I_{OUT} port of the bath-clamp headstage is connected to an indirectly-grounded platinum wire. This configuration requires that the V_m electrode is connected to the voltage probe input of the oocyte clamp (channel "V" in Figure 7b) rather than to channel B of the differential electrometer. Thus the intracellular ion-activity is proportional to the difference in outputs between Channel A and Channel V and is not calculated by the FD223 electrometer. Instead, A–V must be calculated separately either by electronic means prior to digitization, or by the acquisition software following digitization (e.g., using the 'Math' facility when setting the acquisition protocol in the Axoscope module of pClamp).

Oocyte impalement with microelectrodes is more easily visualized if the dark animal pole of the oocyte is facing upwards. The V_m electrode is first introduced into the oocyte to measure the resting membrane potential of the cell. For a healthy H_2O -injected oocyte, the resting potential should be more negative than -50 mV. The V_m of oocytes expressing a channel or transporter will vary depending on the ion transporting properties of the protein being overexpressed. The second impalement of the oocyte is with the ISM and may cause a transient V_m depolarization. However, if the cell is healthy, the membrane will re-seal around the ISM tip and the V_m will return to its pre-impalement value in a matter of minutes. Once stable V_m and stable ISM values have been achieved, experimental data can be acquired. Example studies that have employed intracellular ISMs in *Xenopus* oocytes can be found in refs. [72, 73, 74, 75, 76, 45, 77, 78].

Typical parameters for control oocytes, determined by ISM measurement using this experimental configuration, are 3–4 mM intracellular Na^+ [74, 77], 24 mM intracellular Cl^- [77], and an average intracellular pH of 7.33 [79].

Surface ISM recordings—For surface ion-selective measurements, it is necessary for both the surface ISM and its reference electrode to be at the surface of the oocyte. We find that KCl-filled electrodes of the type discussed above are unsuitable as a reference for surface ISM recordings. Typically we are measuring small changes in ion activities at the cell surface so it is vital that the reference electrode is as "drift-free" as possible. Thus we use a miniature calomel reference electrode (Cat.No.#13-620-79; Thermo Fisher Scientific Inc.) connected to the input ("Channel C" in Figure 7c) of an electrometer such as a Model 750C or FD223 (World Precision Instruments Inc.). Stable readings from a calomel electrode, a mercury/mercurous-chloride half-cell, typically deviate by less than 2 mV from the mean. In order to bridge the calomel electrode to the chamber solution surrounding the oocyte, the calomel is placed in a custom-made holder that is filled with 3 M KCl. The holder is contiguous with a long, 3 M KCl-filled, broken-tipped glass micropipette^m, the tip of which is positioned in the chamber solution close to the oocyte surface. Figure 7c represents a configuration that can be used to simultaneously monitor ion activity at the oocyte surface (proportional to the differential output "Channel B–Channel C"), ion activity in the oocyte cytoplasm (proportional to the differential output "Channel A–Channel V"), and oocyte V_m (the output of Channel V). The positioning

^mThe elongated micropipette is pulled from thin-walled borosilicate tubing on a rotating micropipette puller (e.g., Model 51.511, Stoelting Co., Chicago, IL).

of the electrodes in relation to the measurement site for this configuration is represented in Figure 8b.

Positioning the blunt tip of a surface ISM at the extracellular surface of the oocyte can be performed using an ultra-fine computer-controlled micromanipulator with digital position display (model MPC-200 system, Sutter Instrument Company, Novato, CA). First, the micromanipulator is used to position the surface ISM very close to the oocyte membrane, until it just touches the surface of the oocyte (the “0” position). Next the ISM is further advanced towards the membrane, until a dimple of ~40 µm from the “0” position is achieved on the oocyte membrane. Periodically during the experiment, the electrode is withdrawn 200–300 µm from the oocyte surface into the bulk chamber solution to check that the electrode calibration has not drifted. Recent examples of studies that have employed surface ISMs with *Xenopus* oocytes can be found in refs. [80,35,81,78,82].

5. CONCLUDING REMARKS

An experiment in which the ion activity of an oocyte is measured using an ISM is not trivial either in terms of set-up or duration so it is critical to be able to identify non-expressing or poorly-expressing oocytes prior to assay. These oocytes, which may be unhealthy, may have been mis-injected, or may have been injected with poor-quality/degraded cRNA can be identified and discarded. Furthermore, the robustness of expression between different groups of oocytes can be determined, allowing for normalization of ISM-measured data between groups and quality control of expression-robustness between different ovary preparations. Thus the ability to non-invasively monitor transporter expression prior to oocyte assay is a useful tool to complement physiological data collection.

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Figure 1. Features of pGH19, a typical ‘oocyte-expression’ plasmid for producing *Xenopus*-expression-ready cRNAs

The plasmid DNA sequence is represented as a circle. Plasmid features are labeled to scale and delineated with colored blocks. Clockwise from the 12-o-clock position the features shown are an M13F promoter sequence (red) for priming DNA sequencing reactions in the direction of the multiple cloning site, a T7 promoter sequence (green) for priming in-vitro transcription reactions, the 5'-UTR of *Xenopus* β -globin cDNA (pink; 5'), a multiple cloning site into which the gene of interest is subcloned, the 3'-UTR of *Xenopus* β -globin cDNA (pink; 3'), a polyadenosine tract (yellow; pA) followed by a polycytosine tract (orange; in pGH19 31 cytosines follow 23 adenosines), the plasmid linearization site, an SP6 (green) and a M13R promoter sequence (red) either of which can be used for priming DNA sequencing reactions in the direction of the multiple cloning site, and the ampicillin resistance gene (purple; AmpR) that allows selection of bacterial transformants during subcloning. The AmpR gene is preceded by its promoter (green). The origin of replication of pGH19 (not shown) is located in the region between AmpR and M13R. The plasmid diagram was generated using BVTech Plasmid software (BV Tech Inc., Bellevue, WA).

Synthesizing Capped cRNA

- Linearize ≥ 5 μg plasmid template DNA using the appropriate restriction enzyme.
- Purify linearized DNA using QIAgen PCR purification kit or QIAgen nucleotide removal kit.
- Resuspend purified, linear DNA in 30 μl RNase free H_2O .
- Set up a 1 to 3 hour in-vitro transcription reaction with Ambion mMessage mMachine kit using 1 μg template DNA.
- Purify cRNA using QIAgen RNeasy MinElute cleanup kit.
- Quantify cRNA by spectrophotometry. (1 OD unit at $A_{260} = 40$ ng/ μl).
- Dilute cRNA to a working concentration (e.g., 1 $\mu\text{g}/\mu\text{l}$) with RNase-free H_2O , divide into working aliquots (e.g., 2 μl), and store frozen at -20° or -80°C until use.

Figure 2. Workflow for capped-cRNA synthesis from a cDNA template

For more detailed considerations of nucleic acid synthesis and purification we refer the reader to the manuals that accompany the kits mentioned in the workflow chart. For cRNA being synthesized for the first time from a new template, we recommend that the size and integrity of the cRNA be confirmed by agarose gel electrophoresis, for example using the Formaldehyde-free RNA Gel Kit (AMRESCO Inc., Solon, OH).

Isolating Defolliculated Oocytes

- Dissolve 60 mg of type IA collagenase in 30 ml 0-Ca⁺⁺ NRS solution. Filter sterilize and pour into a sterile Petri dish.
- Using sterile scissors, cut the oocyte lobes into $\leq 5 \text{ mm}^3$ pieces and drop directly into the collagenase solution.
- Shake the dish containing oocytes on a rocking platform for 30-60 min. Monitor the digestion status by regularly transferring a few oocytes into ND96 solution and visualizing under a dissecting microscope. Oocyte digestion should be continued until the cells are mostly separated from each other and have lost their follicle-cell layer, which is glossy and vascular in appearance.
- Vigorously rinse the oocytes several times in 0-Ca⁺⁺ NRS solution and shake for 15 min.
- Vigorously rinse the oocytes several times in ND96 and shake for a further 15 min.
- Transfer the oocytes into OR3 medium and store at 18°C in a chiller cabinet.

Figure 3. Workflow for isolating defolliculated oocytes

Recipes for the solutions used in these procedures are provided in Table 1. This protocol uses collagenase type 1A (Cat. No. C2674, Sigma-Aldrich). Prior to use, collagenase solution is filter-sterilized using a SteriFlip vacuum filtration unit with a 0.22 μm filter (Cat.No. SCGP00525; Millipore, Billerica, MA).

Figure 4. Examples of useful and non-useful oocytes

(a) Useful oocytes. Oocytes in this category are of a uniform size (~1 mm diameter) and shape with clear separation of color between the dark and light poles. The darkness of the pigmentation at the animal pole varies between frogs. The oocytes shown here are Dumont stage V. (b) Non-useful oocytes. Oocytes in this category are those which are either small, misshapen, discolored or that retain their follicle-cell layer (magnified in inset). The follicle-cell layer is vascular in appearance and gives the cells a shiny appearance. The scale bar applies to panels a and b, but not to the magnified inset in b.

Figure 5. Live control oocytes or live oocytes expressing EGFP visualized by confocal microscopy
 (a) Oocytes are viewed in a focal plane (FP₁) close to the coverslip upon which they are resting. (b) Control oocytes that were injected with H₂O 4 days earlier are viewed by differential interference contrast microscopy. (c) The same oocyte from panel B is now viewed in FP₁ by epifluorescent microscopy (excitation 488 nm/emission collected at 510 nm) revealing a lack of intrinsic fluorescence in this cell. (d) An oocyte expressing NBCe1-EGFP-Ct exhibits substantial fluorescence in FP₁. (e) An oocyte expressing soluble EGFP also exhibits substantial fluorescence in FP₁. The difference in the diameter of the fluorescent areas in panels d and e reflects the area of oocyte membrane that is in contact with the coverslip. (f) Oocytes are viewed in a focal plane (FP₂) close to the equator of the cell. (g) Control oocytes that were injected with H₂O 4 days earlier are viewed by differential interference contrast microscopy. (h) The same oocyte from panel b is now viewed in FP₂ by epifluorescent microscopy revealing a lack of intrinsic fluorescence in this cell. (i) An oocyte expressing NBCe1-EGFP-Ct exhibits substantial fluorescence around its perimeter in FP₂. (j) An oocyte expressing soluble EGFP also exhibits substantial fluorescence around its perimeter in FP₂. All images were acquired using an Olympus Fluoview™ FV1000 confocal microscope.

Figure 6. Live control oocytes or live oocytes expressing EGFP visualized by variable mode imager Oocytes were visualized in situ in their culture dish using a Typhoon imager (excitation 488 nm/emission collected using a 520-nm band-pass filter) (a) Oocytes expressing NBCe1-EGFP-Ct (b) oocytes expressing soluble EGFP, (c) H₂O-injected cells, (d) an empty well containing only OR3 medium. Darker regions correspond to more intense fluorescence. Each oocyte expressing EGFP is thus visualized as a dark circle. Oocytes not expressing EGFP are visualized as lighter circles. The image was acquired at a resolution of 100 $\mu\text{m}^2/\text{pixel}$ at a focal plane 3 mm above the imager platen. The average fluorescence intensity of the oocyte populations in this image is shown in column 3 of Table 2. (e) The oocytes from A-C were transferred into corresponding individual rows (i-iv) of a 96-well plate containing 300 μl of

ND96 and re-imaged. Row iv contains only ND96. This fluorescence of the oocytes in this plate was measured by plate reader (see column 2 of Table 2).

Figure 7. Electronic configurations for acquiring ISM data

(a) A basic configuration in which both the ISM and its KCl-filled reference (Ref.) are connected to channels A and B of an FD223 dual-channel electrometer. Ion activity is proportional to the differential output A-B. The chamber solution is directly grounded via a Pt wire (b) A configuration for simultaneous monitoring of intracellular (Int.) ion-activity and membrane potential. The ISM is connected to channel A of the FD223 while its KCl-filled reference (V_m) is connected to "channel V" of an oocyte clamp. Both electrodes are introduced into an oocyte. The chamber solution is indirectly grounded via a bath-clamp headstage. A Pt wire connected to the I_{OUT} (I_O) port of the bath-clamp headstage holds the chamber potential at 0 mV, the potential sensed by the extracellular KCl-filled reference electrode (V_{ch}) connected to the I_{SENSE} (I_S) port of the bath clamp headstage. Intracellular ion-activity is proportional to the differential output (A-V) and the membrane potential is reported directly by the output of channel V (technically $V-I_S$, where $I_S = 0$ mV). (c) A configuration for simultaneous monitoring of intracellular ion-activity, surface (Sur.) ion- activity and membrane potential. The intracellular ISM is connected to channel A of the FD223 while its KCl-filled reference (V_m) is connected to "channel V" of an oocyte clamp. The surface ISM is connected to channel B of the FD223 while its calomel reference electrode (Cal.) is connected to "channel C" of a second electrometer. The chamber solution is indirectly grounded via a bath-clamp headstage that holds the chamber potential at 0 mV. Intracellular ion-activity is proportional to the differential output (A-V), surface ion-activity is proportional to the differential output (B-C), and the membrane potential is reported directly by the output of channel V (technically $V-I_S$, where $I_S = 0$). In this figure, KCl-filled electrodes are represented as light blue wedges and ISMs are represented as green wedges. In configurations (b) and (c) only the V_m and ISM (Int.) electrodes are introduced into the oocyte.

Figure 8. Representation of the placement of electrodes around an oocyte in the recording chamber
 The recording chamber is represented in gray with the perfusion channel colored blue. The direction of chamber perfusion is indicated with red arrows. The black cross next to the oocyte represents crossed nylon-filaments that can be used to hold the oocyte in position during perfusion. V_m is the KCl-filled reference electrode for the intracellular ISM, V_{ch} is the KCl-filled reference electrode for the V_m electrode. (a) The placement of electrodes used for simultaneous measurement of intracellular ion-activity and membrane potential in an oocyte with the electronic configuration presented in Figure 7b. (b) The placement of electrodes used for simultaneous recording of surface ion-activity, intracellular ion-activity and membrane potential with the electronic configuration presented in Figure 7c.

Table 1

Solutions for the isolation and culture of defolliculated oocytes.

Solution Name	Composition ^a
ND96	93.5 mM NaCl, 2 mM KCl, 1.8 mM CaCl ₂ , 2 mM MgCl ₂ , 5 mM HEPES. Adjust pH to 7.50 with NaOH (contributing an additional 2.5 mM Na to the final solution). Osmolarity should be 195±5 mOsm.
0-Ca ⁺⁺ NRS	82 mM NaCl, 2 mM KCl, 20 mM MgCl ₂ , 5 mM HEPES. Adjust pH to 7.45 with NaOH.
OR3	Add 1 sachet of powdered GIBCO Leibovitz's L-15 medium with glutamine (Cat.No.# 41300-039; Invitrogen, Grand Island, NY) to 1.5 L double distilled H ₂ O. Add 200 U Penicillin, 2000 µg Streptomycin and 1.785 g HEPES. Adjust pH to 7.50 with NaOH and adjust volume with H ₂ O until osmolarity is 195 ± 5 mOsm. Final volume should be ~1800 ml.

^a All solutions are filter sterilized using vacuum-driven filter units such as 0.22 µm PES Steritop/Stericup units (Millipore, Billerica, MA) and stored at 4 °C.

Table 2

Fluorescence intensity of live control oocytes or live oocytes expressing EGFP assayed by plate reader and variable mode imager

Oocyte Group	Plate Reader ^a	Imager ^b
H ₂ O-injected	-95 ± 248	2277 ± 24
NBCe1 EGFP-Ct	4890 ± 529	9316 ± 1080
Soluble EGFP	3343 ± 413	6880 ± 540

^aThese data are plate reader measurements from the cells in Figure 6e.

^bThese data are extracted from the image presented in Figure 6a–d.

Table 3

Commonly used ion-selective cocktails and their accompanying backfill solutions.

Ion	Cocktail (Sigma-Aldrich Catalog Number)	Backfill solution ^a
Cl ⁻	Cl ⁻ liquid ion-exchanger microelectrode cocktail A (#24899)	150 mM NaCl, 5 mM HEPES
H ⁺	H ⁺ -ionophore I - cocktail A (#95293)	40 mM KH ₂ PO ₄ , 23 mM NaOH, 15 mM NaCl
K ⁺	Potassium Ionophore I - cocktail A (# 60031)	15 mM NaCl, 40 mM K ₂ PO ₄ , 23 mM NaOH
Na ⁺	Na ⁺ -ionophore II - cocktail A (#71178)	10 mM NaCl

^a Backfill solutions should be adjusted to pH 7.0 with NaOH and filtered to remove particulate matter by passing through a 0.45µm syringe filter unit (Millipore, MA)