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# The *Xenopus* oocyte: System for the study of functional expression and modulation of proteins

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## Introduction

Why is the *Xenopus* oocyte relevant for food technology? What can it offer? The oocyte from *Xenopus laevis* is an often-used functional expression system, especially for plasma membrane proteins. It allows the transformation of genetic information in the form of DNA or RNA into the encoded protein whose function and the modulation of its function can then be studied. On one hand it can serve to help in the characterization of transport systems for nutritional components or their breakdown products. On the other hand it can serve as a model system for the study of modulation of a defined protein function by food ingredients or its metabolites. The *Xenopus* oocytes can easily be obtained from female animals kept in the laboratory. Proteins of interest are expressed in this system that is easily accessible to experimentation.

Beside this specific application of the expression system for food technologists, a variety of additional uses of the *Xenopus* oocyte is reviewed. Major applications are structure-function studies where the consequence of changes at the level of molecular biology are observed at the level of protein function. An extension is the introduction of unnatural amino acid residues into proteins. Many receptors and carriers have been identified by the so-called expression cloning, whereby a specific function leads to identification of the cDNA coding for the protein responsible for this function. Also the *Xenopus* oocyte has contributed to our understanding of genetic diseases, where an abnormal protein carries a point mutation. The function of such mutated proteins has been compared with the "healthy" parent protein. Additional useful information about the use of the *Xenopus* oocyte as an expression system may also be obtained elsewhere [1, 2].

The review of the applications of the *Xenopus* oocyte will be preceded by the natural history of the frog *Xenopus laevis*, by the discussion of the practical steps involved in the isolation of the oocytes and their microinjection with genetic information in the form of nucleic acids, and the problems of detection of a newly expressed protein. Limitations of this expression system will also be discussed.

## History of the *Xenopus* oocyte

Wild South-African clawed frogs *Xenopus laevis* are living in ponds or dead river arms. The oocytes are deposed in an unfavorable environment. Probably for this reason, they are resistant cells that can be easily handled in the laboratory. They are fully provided with all the organelles, nutriments, enzymes, and substrates required in their early stage of development following fertilization. In fact, its size remains unaltered up to the 1024-cell stage. This total autonomy makes each oocyte an independent factory able to manufacture new proteins. The surface membrane is relatively poor in endogenous ion channels [3], a property that makes the oocyte an attractive expression system for ion channels.

The *Xenopus* oocyte as an expression system for proteins (Fig. 1) has been discovered by Gurdon *et al.* [4]. These authors demonstrated that microinjection of genetic information in the form of mRNA coding for the human protein globin into the cytoplasm of the oocyte results in the synthesis of human globin. Later it was shown that injection of the corresponding coding DNA into the nucleus also resulted in the synthesis of human globin [5]. Thus, transcription and translation of foreign genetic information can be performed by the *Xenopus* oocyte. Not only synthesis of

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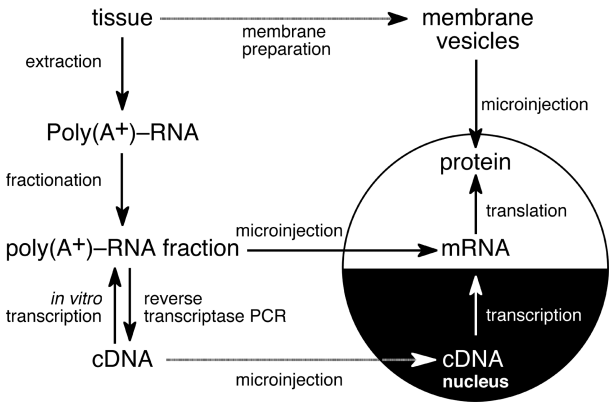


**Figure 1.** Scheme of the *Xenopus* follicle showing microinjection of mRNA, cDNA, or a cell membrane suspension into the oocyte.

foreign cytoplasmic proteins is possible. Sumikawa *et al.* [6] showed that injection of mRNA coding for nicotinic acetylcholine receptor results in the formation of radioactive ligand binding sites in the oocyte surface membrane indicative of the new synthesis of the corresponding protein. A year later it was shown that the oocyte plasma membrane acquired indeed novel electrophysiological properties demonstrating for the first time functional expression of a plasma membrane protein [7]. The most important early application of the *Xenopus* oocyte expression system was to verify that an mRNA or cDNA is coding for the expected protein function [8]. Expression of plasma membrane protein in oocytes may also be achieved by injection of the mature protein. Thus, it was shown that microinjection of a membrane preparation containing the nicotinic acetylcholine receptor results in the incoporation of these receptors into the surface membrane [9].

## Practical Procedures

### Description of *Xenopus* oocytes

In adult female, oogenesis is asynchronous. This means that the six stages of oocyte development occur at the same time. However, ovaries of adult frogs mainly contain stage V and VI oocytes [2]. The fully grown stages V or VI oocyte are the preferred cells for expression studies. These stage V or VI oocytes are large round cells (1.1–1.2 mm diameter) with a characteristic appearance: the cell is divided in a dark-brown ("animal") hemisphere and a yellowish ("vegetal") hemisphere. The animal hemisphere, whose color is due to the high concentration of melanin below the surface membrane, contains the nucleus. The oocyte is surrounded by different layers of cellular and noncellular material. Closest to the plasma membrane of the cell is the vitelline membrane, which is a noncellular fibrous layer. Then there is a layer of follicle cells electrically connected to the oocyte by gap junctions, a connective tissue layer, and an epithelial cell layer relying the ovary wall. We call this complete structure in the following "follicle".

### Preparation of *Xenopus* oocytes

Oocytes which have reached stage VI remain at this point for several months. For this reason, it is possible to remove oocytes several times from the same female by waiting few weeks between two subsequent surgeries. In addition, removal of oocytes seems to stimulate formation of new oocytes. We describe here the protocol used routinely in our laboratory. For alternative procedures used in many other laboratories see also [2].

Female *Xenopus laevis* are kept under a 12 h day/night cycle. Other conditions are described in several links of www.xenopus.com/links. The animals are anesthetized by immersion until loss of all reflexes (~10–15 min) in prechilled water containing 2% ethyl 3-aminobenzoate methane sulfate (A5040; Sigma, St. Louis, MO, USA). The female is then laid on wet tissues placed on an ice bed (ventral face up) and kept wet by covering it with soaked tissue. The nose of the animal must be exposed to air to enable breathing. Through a small abdominal incision (0.5–0.8 cm) lobes of the ovary are pulled out carefully (Figs. 2, 3). At least two but not all lobes of the ovary should be removed to ensure oocyte regeneration. Follicles are mechanically singled out from an ovary lobe using a platinum loop (Fig. 4). Follicles (Fig. 5) are then stored at 18°C in sterile filtered Barth' medium containing NaCl (88 mM), KCl (1 mM), $NaHCO_3$ (2.4 mM), HEPES (10 mM, pH 7.5), $MgSO_4 \cdot 7H_2O$ (0.82 mM), $Ca(NO_3)_2 \cdot 4H_2O$ (0.34 mM),

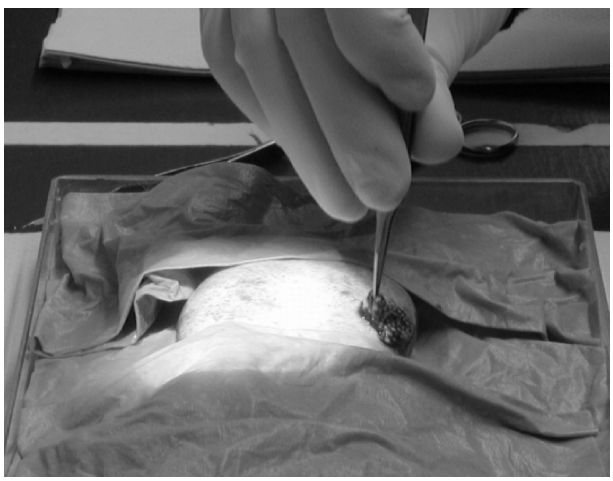

**Figure 2.** Surgical removal of an ovary lobe from the abdomen of a female frog *Xenopus laevis*.

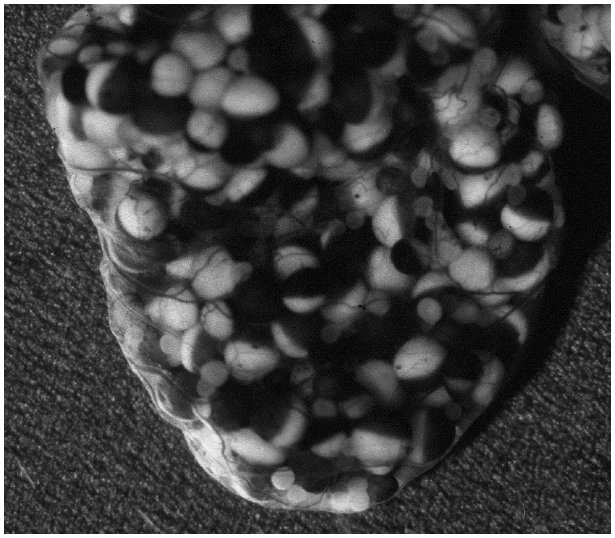

**Figure 3.** The ovary lobe is wrapped into connective tissue and contains the follicles.

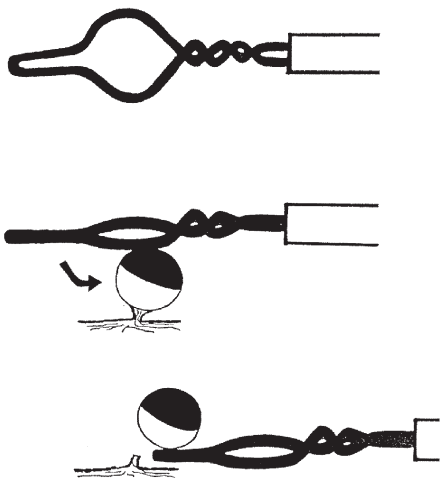

**Figure 4.** Schematic representation of the use of a platinum loop to mechanically isolated single follicles from the ovary lobe.

$CaCl_2 \cdot 2H_2O$ (0.41 mM), and penicillin/streptomycin (100 U/mL).

### Injection of RNA and peeling of *Xenopus* oocytes

Microinjection of cDNA results in many laboratories only in protein expression in a small percentage of the injected oocytes. Therefore, we restrict ourselves to expression of RNA that yields successful expression in 100% of the injected cells. In the case of recombinant proteins, cRNA

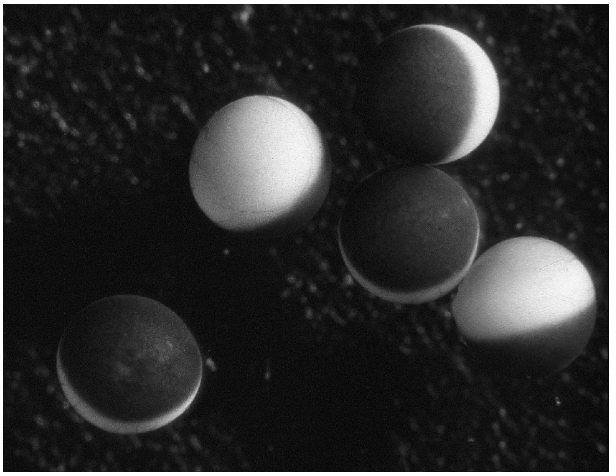

**Figure 5.** Singled-out follicles. Blood vessels can be seen in the connective tissue surrounding each oocyte.

may be prepared from cDNA by *in vitro* transcription, poly($A^+$)-addition and RNA quantification on gel. Follicles are microinjected with a volume of 50 nL RNA solution with a paraffin oil-filled [10] glass pipette of about 12–15 μm opening diameter (Fig. 6). The amount of RNA varies from about 5 μg/mL for a cRNA coding for an efficiently expressing recombinant protein to about 1.5 mg/mL for total mRNA isolated from a tissue. This upper limit is imposed by the viscosity of concentrated RNA solution which makes squeezing through the injection pipette tip difficult. After injection follicles are stored at 18°C. To obtain naked or denuded oocytes, the surrounding layers, except for the vitelline layer that provides mechanical stability without preventing access to the oocyte plasma membrane, are removed (Fig. 7) [11]. For this purpose, follicles are exposed for 20 min at 36°C to ~1 mg/mL collagenase (Type IA, C-9891, 800 U/mL; Sigma)/0.1 mg/mL trypsin inhibitor (Type I-S, Sigma T-9003) in Barth's solution in borosilicate glass tubes. Subsequently, follicles are exposed for 4 min at room temperature to a doubly concentrated Barth's solution containing 4 mM Na-EGTA. Oocytes may then be conveniently freed from the surrounding layers by rolling them in a plastic culture dish. The newly expressed protein can then be detected after about 20 h in the case of cRNA injection and after about 2–4 days in the case of total mRNA injection.

### Detection of newly expressed proteins

If the newly expressed protein function is endogenously present, the protein in question has to be expressed at a level exceeding the endogenous level. Fortunately for most expressed protein functions, identical endogenous functions are not present, making detection more simple. Newly

 

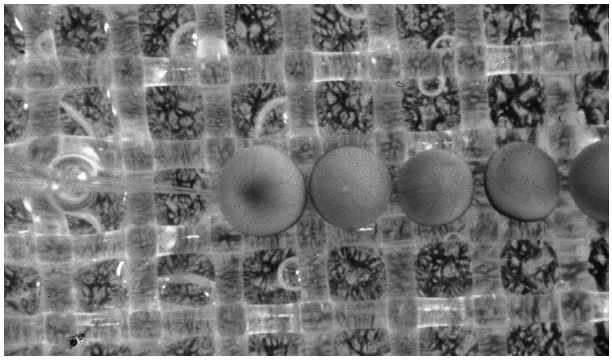

**Figure 6.** Follicle microinjection. The glass needle on the left is filled with RNA solution.

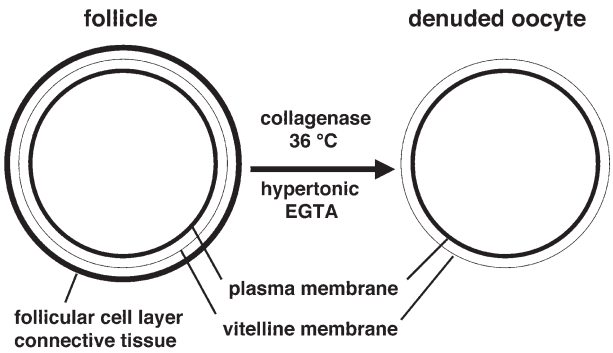


**Figure 7.** Scheme showing the surrounding layers of a follicle and a denuded oocyte. Connective tissue and follicle cells are removed from the follicle to give the denuded oocyte.

expressed proteins have been detected functionally by determining the corresponding activity in the case of enzymes. Structural detection in oocyte detergent extracts may rely on immunodetection in Western blots. If prior immunoprecipitation is possible, problems with egg yolk contamination of oocyte extracts are decreased. An elegant detection is possible if the protein in question is fused to a fluorescent protein. For example, expression of the γ-amino-*n*-butyric acid type A ($GABA_A$) receptor has been detected as green fluorescence in the surface membrane after tagging of the $\alpha_1$-subunit with the green fluorescent protein [12]. Fluorescent detection has also been performed using immunological techniques directly on living oocytes [12]. A fluorescent protein absorbing at long wavelength is preferable as problems with autofluorescence of the oocyte are decreased in this case. A very sensitive detection is provided by metabolic labeling followed by immunoprecipitation. For example, the subunits of the $GABA_A$ receptor have been metabolically labeled using [$^{35}$S]methionine followed by immunoprecipitation exclusively from the surface membrane in order to establish the subunit stoichiometry [13]. As the oocyte is osmotically quite stable, novel expression of water channels may be detected by the acquisition of osmotic sensitivity detected by swelling of the oocyte in diluted medium. For the detection of electroneutral carriers, isotope flux techniques are conveniently used. In case the carriers mediate electrogenic flux, novel expression may be monitored by current measurement using electrophysiological techniques. The latter techniques have also extensively been used for the detection of newly expressed ion channels. In this case, a very sensitive detection is possible as the turn-over of an ion channel is with about $10^8$/s much higher than for an average enzyme with about $10^2$–$10^4$/s. A very elegant indirect detection technique using endogenous amplification of the signal has been used in the case of receptors activating phospholipase C. The released $IP_3$ results in the release of $Ca^{2+}$ from the oocyte endoplasmic reticulum which in turn activates a $Ca^2$-activated $Cl^-$ channel also endogenous to oocytes. The resulting current is detected by electrophysiological means. For a more detailed discussion of detection of newly expressed proteins in *Xenopus* oocytes and specifically of their post-translational modification see also the article by Soreq [14].

## Application of the *Xenopus* oocyte expression system

From the above discussion it is understandable that the oocyte has found most application for functional expression in the fields of ion channel and carrier research. In the following, we discuss general principles and illustrate them with a randomly chosen example known to us.

### Functional expression

Functional expression of total mRNA isolated from a given tissue may be achieved. In order to demonstrate expression of specific genes in a tissue of interest, total mRNA has been isolated from it and microinjected into *Xenopus* oocytes. This results in the expression of a multitude of new proteins in the oocyte where they can be characterized in detail. An example is the injection of RNA from human brain that resulted in the expression of many ligand- and voltage-gated ion channels [15]. Expression of total mRNA also allows comparison on the abundance of a specific RNA in different tissues. In the case the protein is encoded by a single species of mRNA, size fractionation may be used to enrich this mRNA.

A more modern application is the verification of the functional identity of a cloned DNA. *In vitro* transcription followed by poly($A^+$)-adenylation results in the corresponding cRNA that can be microinjected into the oocyte. In the case of an oligomeric protein, this may not result in function. A combination of the correct cRNAs will then have to be

 

used. An early demonstration for a single subunit protein can be found in the functional expression of the voltage-gated $Na^+$-channel, where injection of a single 7 kb RNA species results in a functional ion channel [16]. In contrast, functional expression of the pentameric nicotinic acetylcholine receptor required injection of three different RNA species [17]. In the case of the pentameric $GABA_A$ receptor, different subunit isoform combinations resulted in different pharmacological properties [18].

## Structure-function studies: point mutation and chimera

In order to pinpoint important amino acid residues, individual residues have been mutated and functional consequences of this change have been monitored. For example, mutation of a specific residue in the $\alpha_1$-subunit of the $GABA_A$ receptor, which is built of five subunits each consisting of more than 200 residues, results in a more than 200-fold decrease in the apparent affinity of the agonist GABA required for opening of the channel [19]. Point mutation of another residue in the same subunit resulted in normal expression of a receptor, but unlike a wild-type receptor the mutated receptor could not be anymore modulated with benzodiazepines [20], a clinically used drug affecting $GABA_A$ receptor function. Positional effects of $GABA_A$ receptor subunits were documented by functional expression and characterization of concatenated subunits, that is subunits linked at the DNA level, thus allowing predetermined positioning of the subunits in the oligomeric protein [21]. In order to pinpoint important residues, often chimeric subunits were engineered between two homologous subunits, one of them conferring a given functional property and the other not. By moving the border of the chimeric subunit, regions responsible for this functional property were identified. This is illustrated in the following [22]. The receptors formed upon co-expression of the two subunits $\alpha_1$ and $\beta_2$ co-expressed with either $\gamma_2$ or $\gamma_3$ differ in their binding properties for the benzodiazepine receptor ligand zolpidem. While $\alpha_1\beta_2\gamma_2$ results in high affinity for this ligand, $\alpha_1\beta_2\gamma_3$ results in low affinity. Eight chimera were produced with an increasing proportion of the $\gamma$-subunit formed by $\gamma_3$. While the chimera consisting of the *N*-terminal 115 residues of $\gamma_3$ and the residual subunit of $\gamma_2$ retained high affinity, the chimera consisting of 133 residues of $\gamma_3$ lost high affinity. Within the region 115–133 only five residues differed between $\gamma_2$ and $\gamma_3$. These residues were mutated individually and this led to the identification of a single residue responsible for the pharmacological difference.

## Structure-function studies: unnatural amino acids

Point mutation studies are restricted to the 20 amino acid residues used by nature resulting in most cases in a drastic chemical change at the site of mutation. Especially in functional studies directed at the binding site of a channel agonist or in catalytic centers of enzymes it is of advantage to vary the chemical properties of the amino acid residue side chains more subtly. This can be achieved by introduction of an unnatural amino acid in a desired position. A cDNA is engineered carrying the rarely used amber stop codon in the desired position. Translation of the corresponding RNA is arrested in this position. Translation may be rescued by microinjection of a chemically engineered t-RNA recognizing the amber codon and being charged with the artificial amino acid. This results in the synthesis of a protein carrying the unnatural amino acid. An example is provided by Nowak *et al.* [23] who were able to subtly vary the $pK_a$ of the residue in position 93 of the α-subunit in the agonist binding pocket of the nicotinic acetylcholine receptor. The natural residue in this position is a tyrosine with a $pK_a$ of 10. Artificial amino acids with a $pK_a$ down to 5 could be introduced here, without altering the agonist properties. This result suggested that the residue in position 93 is protonated even if the $pK_a$ of the free amino acid would predict a charged residue.

## Characterization of *in vivo* mutated proteins: channelopathies

Many inherited diseases are caused by mutation of a protein (point mutation, expansion of nucleotide repeats, deletion). Especially in the case of ion channels, the *Xenopus* oocyte expression system has been used to study functional consequences of such disease mutations. Wallace *et al.* [24] demonstrated that, in the generalized epilepsy with febrile seizures plus ($GEFS^+$) disorder, a mutation in the voltage-gated sodium ($Na^+$)-channel $\beta_1$-subunit gene (SCN1B), when co-expressed with a brain $Na^+$-channel α-subunit in the *Xenopus* oocyte, interferes with the ability of the subunit to modulate channel-gating kinetics. Another example is provided by the case of spinocerebellar ataxia type 6 (SCA6). In this dominantly inherited degenerative disorder of the cerebellum the underlying mutation consists of an expansion of a trinucleotide CAG repeat in the 3′-region of the gene encoding the $\alpha_{1A}$-subunit of the neuronal P/Q-type voltage-gated calcium channel. Using $\alpha_{1A}$-subunit chimeras including SCA6 mutations, it was shown that the SCA6 polyglutamine expansion shifts the voltage dependence of channel activation and rate of inactivation, when expressed with $\beta_4$ subunits and impairs normal G-protein regulation of P/Q channels [25]. For an exhaustive review on ion channel diseases see [26].

## Modulation of protein function by small molecules

Often a protein function is not easily accessible to experimentation. Transplantation of this protein into the oocyte is

 

one of the possibilities to enable investigation. An example is provided by the flavone hispidulin (4′,5,7-trihydroxy-6-methoxyflavone) that was isolated from sage (*Salvia officinalis* L.) and identified as a potent ligand of the central human benzodiazepine receptor. This pointed to the $GABA_A$/benzodiazepine receptor but radioactive ligand binding studies are usually not able to predict functional effects of a compound. Therefore, effects of hispidulin were investigated at recombinant $GABA_A$/benzodiazepine receptors expressed by *Xenopus laevis* oocytes [27]. Concentrations of 50 nM and higher stimulated the GABA-induced chloride currents at all receptor subtypes ($\alpha_{1\text{-}3,5,6}\beta_2\gamma_2$) indicating positive allosteric properties. In contrast to diazepam, hispidulin exhibited affinity to the $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptor subtype.

### Functional expression cloning

As mentioned above, injection of mRNA isolated from a whole tissue results in the expression of a diversity of protein functions. Functional expression cloning provides a possibility to isolate the cDNA coding for one of these protein functions [28]. First, tissues are screened for the highest content of the RNA coding for the desired function. Second, a size fractionation of the corresponding total mRNA is performed. The size fraction coding for the desired function is converted into a cDNA library by reverse transcription. After size fractionation of the cDNA library, the library is progressively subdivided until a single clone has been identified that codes for the desired function. Numerous carriers and receptors linked to phospholipase C have been cloned using this technique. The earliest use of the oocyte as expression cloning system was by Hediger *et al.* [29] who identified the cDNA coding for the $Na^+$/glucose transporter in rabbit small intestine. Please note that expression cloning is well-suited for proteins that are encoded by a single species of DNA. Procedures may be adapted to other cases but as a consequence become very complex.

## Limitations of the *Xenopus* oocyte expression system

Many laboratories observe a strong and sometimes seasonal variability in the quality of oocytes and are often limited to work within the first 1–3 days after surgery. This is most probably due to the damaging standard procedure of oocyte isolation that subjects the ovary lobes directly to several hours of a collagenase treatment. While this limitation may easily be circumvented, there are true limitations to the use of *Xenopus* oocytes. This is the case where huge amounts of recombinant protein must be obtained, for example, in studies of protein crystallization. Due to the rather small number of cells involved, the system may only be used for radioactive ligand binding studies in cases where a ligand of high-specific activity is available. Also when stable expression is required, the *Xenopus* oocyte is obviously not suitable. Due to its huge size the oocyte poses some limitations in the required times for a change in membrane potential or for a solution change. A conventional electrophysiological setup (2-electrode-voltage-clamp) takes about 2 ms to settle to a new membrane potential, far longer than the time period required to open a fast voltage-gated ion channel. A solution change on a conventional setup takes about 1 s, far longer than the millisecond time scale in which synaptic events between two neurons take place. Therefore, the *Xenopus* oocyte in combination with a conventional setup is not the appropriate expression system for the study of very fast events. However, both time limitations may be avoided using the patch-clamp technique.

## Conclusions

The *Xenopus* oocyte is a very often used functional expression system. It has been in use since its discovery more than 30 years ago. As judged from publications that have been published during the past few years, it appears still as one of the standard expression systems. In the new environment of the oocyte, proteins which are *in situ* often very difficult to access become easily amenable to experimentation. The most attractive applications in food technology include characterization of transport systems for food ingredients or their breakdown products (see, for example, [29]) and generally modulation of protein function by these molecules (see, for example, [27]).

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## References

[1] Sigel, E., Use of *Xenopus* oocytes for the functional expression of plasma membrane proteins. *J. Membr. Biol.* 1990, *117*, 201 – 221.
[2] Wagner, C. A., Friedrich, B., Setiawan, I., Lang, F., Broer, S., The use of *Xenopus* laevis oocytes for the functional characterization of heterologously expressed membrane proteins. *Cell. Physiol. Biochem.* 2000, *10*, 1 – 12.
[3] Dascal, N., The use of *Xenopus* oocytes for the study of ion channels. *CRC Crit. Rev. Biochem.* 1987, *22*, 317 – 387.
[4] Gurdon, J. B., Lane, C. D., Woodland, H. R., Marbaix, G., Use of frog eggs and oocytes for the study of messenger RNA and its translation in living cells. *Nature* 1971, *233*, 177 – 182.
[5] Mertz, J. E., Gurdon, J. B., Purified DNAs are transcribed after microinjection into *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* 1977, *74*, 1502 – 1506.

 

[6] Sumikawa, K., Houghton, M., Emtage, J. S., Richards, B. M., Barnard, E. A., Active multi-subunit ACh receptor assembled by translation of heterologous mRNA in *Xenopus* oocytes. *Nature* 1981, *292*, 862 – 864.

[7] Barnard, E. A., Miledi, R., Sumikawa, K., Translation of exogenous messenger RNA coding for nicotinic acetylcholine receptors produces functional receptors in *Xenopus* oocytes. *Proc. R. Soc. Lond. B* 1982, *215*, 241 – 246.

[8] Rungger, D., *Xenopus helveticus*, an endangered species? *Int. J. Dev Biol.* 2002, *46*, 49 – 63.

[9] Marsal, J., Tigyi, G., Miledi, R., Incorporation of acetylcholine receptors and $Cl^-$ channels in *Xenopus* oocytes injected with Torpedo electroplaque membranes. *Proc. Natl. Acad. Sci. USA* 1995, *92*, 5224 – 5228.

[10] Colman, A., in: Hames, B. D., Higgings, S. J. (Eds.), Transcription and Translation. A Practical Approach. IRL, Oxford-Washington, 1984, pp. 271 – 302.

[11] Sigel, E., Properties of single sodium channels translated by *Xenopus* oocytes after injection with messenger ribonucleic acid. *J. Physiol. (Lond.)* 1987, *386*, 73 – 90.

[12] Connor, J. X., Boileau, A. J., Czajkowski, C., A $GABA_A$ receptor $\alpha_1$ subunit tagged with green fluorescent protein requires a β-subunit for functional surface expression. *J. Biol. Chem.* 1998, *273*, 28906 – 28911.

[13] Kellenberger, S., Eckenstein, S., Baur, R., Buhr, A., Malherbe, P., Sigel, E., Subunit stoichiometry of oligomeric membrane proteins: $GABA_A$ receptors isolated by selective immunoprecipitation from the cell surface. *Neuropharmacology* 1996, *35*, 1403 – 1411.

[14] Soreq, H., The biosynthesis of biologically active proteins in mRNA-microinjected *Xenopus* oocytes. *CRC Crit. Rev. Biochem.* 1985, *18*, 199 – 238.

[15] Gundersen, C. B., Miledi, R., Parker, I., Messenger RNA from human brain induces drug- and voltage-operated channels in *Xenopus* oocytes. *Nature* 1984, *308*, 421 – 424.

[16] Noda, M., Ikeda, T., Suzuki, H., Takeshima, H., *et al.*, Expression of functional sodium channels from cloned cDNA. *Nature* 1986, *322*, 826 – 828.

[17] Mishina, M., Kurosaki, T., Tobimatsu, T., Morimoto, Y., *et al.*, Expression of functional acetylcholine receptor from cloned cDNAs. *Nature* 1984, *307*, 604 – 608.

[18] Sigel, E., Baur, R., Trube, G., Möhler, H., Malherbe, P., The effect of subunit combination of rat brain $GABA_A$ receptors on channel function. *Neuron* 1990, *5*, 703 – 711.

[19] Sigel, E., Baur, R., Kellenberger, S., Malherbe, P., Point mutations affecting antagonist affinity and agonist dependent gating of $GABA_A$ receptor channels. *EMBO J.* 1992, *11*, 2017 – 2023.

[20] Buhr, A., Schaerer, M. T., Baur, R., Sigel, E., Residues at positions 206 and 209 of the $\alpha_1$-subunit of γ-aminobutyric $acid_A$ receptors influence affinities for benzodiazepine binding site ligands. *Mol. Pharmacol.* 1997, *52*, 676 – 682.

[21] Minier, F., Sigel, E., Positioning of α-subunit isoforms confers a functional signature to $GABA_A$ receptors. *Proc. Natl. Acad. Sci. USA* 2004, *101*, 7769 – 7774.

[22] Buhr, A., Sigel, E., A point mutation in the $\gamma_2$-subunit of γ-aminobutyric acid type A receptors results in altered benzodiazepine binding site specificity. *Proc. Natl. Acad. Sci. USA* 1997, *94*, 8824 – 8829.

[23] Nowak, M. W., Kearney, P. C., Sampson, J. R., Saks, M. E., *et al.*, Nicotinic receptor binding site probed with unnatural amino acid incorporation in intact cells. *Science* 1995, *268*, 439 – 442.

[24] Wallace, R. H., Wang, D. W., Singh, R., Scheffer, I. E., *et al.*, Febrile seizures and generalized epilepsy associated with a mutation in the $Na^+$-channel $\beta_1$-subunit gene *SCN1B*. *Nature Genetics* 1998, *19*, 366 – 370.

[25] Restituito, S., Thompson, R. M., Eliet, J., Raike, R., *et al.*, The polyglutamine expansion in spinocerebellar ataxia type 6 causes a β-subunit-specific enhanced activation of P/Q-type calcium channels in *Xenopus* oocytes. *J. Neurosci.* 2000, *20*, 6394 – 6403.

[26] Lehmann-Horn, F., Jurkat-Rott, K., Voltage-gated ion channels and hereditary disease. *Physiol. Rev.* 1999, *79*, 1317 – 1372.

[27] Kavvadias, D., Sand, P., Youdim, K. A., Qaiser, M. Z., Rice-Evans, C., Baur, R., Sigel, E., Rausch. W. D., Riederer, P., Schreier, P., The flavone hispidulin, a benzodiazepine receptor ligand with positive allosteric properties, traverses the blood-brain barrier and exhibits anticonvulsive effects. *Br. J. Pharmacol.* 2004, *142*, 811 – 820.

[28] Romero, M. F., Kanai, Y., Gunshin, H., Hediger, M. A., Expression cloning using *Xenopus laevis* oocytes. *Methods Enzymol.* 1998, *296*, 17 – 52.

[29] Hediger, M. A., Coady, M. J., Ikeda, T. S., Wright, E. M. Expression cloning and cDNA sequencing of the $Na^+$/glucose co-transporter. *Nature* 1987, *330*, 379 – 381.

 