Chapter 3

In-Cell NMR in *Xenopus laevis* Oocytes

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Abstract

For the purpose of studying IDPs inside cells of higher organisms, several eukaryotic in-cell NMR systems have been developed over the past years. In this chapter we will focus on high-resolution in-cell NMR applications in *Xenopus laevis* oocytes, the first eukaryotic cellular model system to be established. In contrast to prokaryotic in-cell NMR samples, eukaryotic in-cell NMR specimens are prepared by cytoplasmic delivery of an exogenously produced, isotope-labeled protein into the non-isotope-labeled environment of the respective "host" cell. In-cell NMR applications in *Xenopus* oocytes rely on intracellular sample deposition by direct microinjection into the oocyte cytoplasm. Here, we describe the preparation of oocyte in-cell NMR samples for IDP studies in this cellular model environment.

Key word: *Xenopus laevis* oocytes

1. Introduction

In-cell NMR applications in eukaryotic cells enable high-resolution investigations of IDPs in cellular environments that exhibit many of the biological activities typically encountered in higher organisms (1). While NMR experiments that are typically employed to study IDPs in vitro are essentially the same than for in-cell NMR studies, Chapters 3–6 outline different protocols for protein delivery into eukaryotic cells, rather than specific NMR methods. These can roughly be divided into two parts: Protein delivery protocols for large amphibian cells (this chapter) and protein transduction methods for very much smaller, mammalian cells (Chapters 4–6). Especially with regard to IDP studies addressing the structural in vivo effects of posttranslational protein modifications (PTMs), eukaryotic in-cell NMR methods offer unique advantages over prokaryotic in-cell NMR measurements. Because the proteins to be delivered into the eukaryotic cellular environment are

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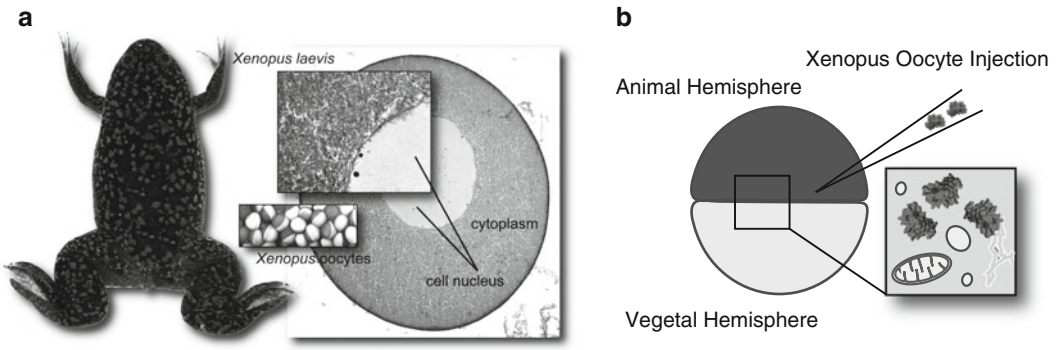

Fig. 1. (**a**) The African clawed frog *Xenopus laevis* and its oocyte cells. A thin-section, low-resolution microscopy image of a single oocyte (HE stained) depicts the large degree of macromolecular crowding in the oocyte cytoplasm. The cell nucleus occupies about 20 % of the total cell volume. The close-up view shows the nuclear envelope boundary that separates the cell nucleus from the cytosol. (**b**) Schematic representation of the spherical structure of the *Xenopus* oocyte. The darkly pigmented animal pole is clearly separated from the brightly colored vegetal hemisphere. Oocyte in-cell NMR samples are prepared by microinjection of isotope-labeled proteins into the cellular cytoplasm.

recombinantly produced in bacteria, they effectively lack the PTMs that are characteristic for many eukaryotic IDPs (2). Upon intracellular sample deposition, endogenous cellular enzymes "realize" this PTM deficiency and actively establish missing modifications, guided by the specific conditions of the respective cellular settings. In this way, IDPs "receive" physiologically relevant sets of PTMs the establishments of which-, and their structural consequences-, are directly observable by time-resolved in-cell NMR experiments (3).

Oocytes from the African clawed frog *Xenopus laevis* have long served as a standard cellular model system in many areas of biology (Fig. 1a) (4). Their large size (~1 μL cell volume) enables the direct delivery of biological compounds such as RNA, DNA or proteins by simple microinjection (Fig. 1b). Because isotope labeling occurs in a different cellular environment than the actual in-cell NMR measurements (i.e., *E. coli* versus *X. laevis* oocytes, respectively), no background labeling artifacts are encountered. While *Xenopus* oocytes may not represent a truly native cellular context for many eukaryotic IDPs, their general physical properties in terms of macromolecular crowding and cellular viscosity closely resemble most other eukaryotic cells. In that sense, crowding induced changes in IDP conformations for example, are likely to also occur in this cellular setting (5). In addition, *Xenopus* oocytes are naturally cell-cycle synchronized. In the ovary lobe of female frogs, oocytes mature into stage VI cells and arrest at the G2/M boundary of the first meiotic division. During oocyte to egg maturation, a hormonal trigger activates the synchronized progression through both meiotic cycles, until all cells arrest in metaphase of meiosis II. Concomitant changes in cellular PTM activities are uniformly displayed in all cells, which allows for "discrete" PTM studies even when multiple

cells are used. Furthermore, concerted cell-cycle progression can be activated in vitro by the addition of the hormone progesterone (PG), which renders this system an important laboratory tool for studying cell-cycle dependent signaling pathways and cellular kinase activities (6). In-cell NMR studies of biomolecules in *Xenopus* oocytes have been performed on folded (3, 7, 8) and natively unfolded proteins (9), as well as on RNA and DNA (10). Detailed descriptions of oocyte in-cell NMR sample preparations have been reported in these papers and also in (11). The goal of this chapter is to provide an updated manual that comprehensively integrates the experimental advances made over the recent years in the preparation of in-cell NMR samples employing *Xenopus laevis* oocytes.

2. Materials

1. Equipment: The protocol assumes that access to *Xenopus laevis* frogs is available and that proper animal care and safety protocols are obeyed at all times. Institutional licenses for housing and manipulating frogs must be in place. A standard laboratory setup for surgically removing oocytes and for manipulating oocytes by microinjection is needed. This includes a glass capillary puller to prepare injection needles (or access to a commercial vendor), a dissecting microscope with a built-in micrometer scale, and a standard pneumatic oocyte injection device (Harvard Instruments). Alternatively, large-scale oocyte manipulations can be performed with fully automated injection robots like the Roboinject™, or Robocyte™ (Multichannel Systems). In addition, standard laboratory equipment for recombinant protein production and purification, including a fast protein liquid chromatography (FPLC) system, is needed. Access to high-field (>500 MHz) solution-state NMR spectrometers must be available.
2. Pregnant mare serum gonadotropin (PMSG, Sigma Aldrich, USA).
3. Anesthetizing buffer, AB: 1 L of 0.25 mM Na_2CO_3 + 1 g of Tricaine mesylate, TMS.
4. OR2 buffer 82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6.
5. ND96 buffer 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2 \times 2 H_2O$, 1 mM $MgCl_2 \times 6H_2O$, 5 mM HEPES, pH 7.6.
6. Collagenase (Sigma Aldrich, USA).

3. Methods

3.1. Recombinant Protein Expression and Purification

1. Express the protein of interest recombinantly, incorporating the NMR-active isotope label or labels. Commonly, the gene encoding the protein of interest is inserted in a T7 inducible-expression vector transformed into BL21 (DE3) *E. coli* cells. Grow bacteria in labeled growth medium to an OD_{600} of 0.6 and induce expression with IPTG (see Note 1).
2. Purify the protein using standard protein chromatography steps (see Note 2).
3. Concentrate the pure protein to ~1 mM using a centrifugal filter unit, or any other appropriate device (see Note 3).

3.2. Oocyte Preparation

1. For microinjections, prime female frogs with 0.5 mL of 200 U/mL PMSG between 2 and 5 days prior to harvesting the oocytes (see Note 4).
2. Anesthetize frogs by placing them in 1 L of AB for 20 min. All subsequent steps of this section should be carried out at 18 °C.
3. Make a small incision through the abdominal tissue and remove the ovary lobes with forceps.
4. Suture the incision closed and allow the frogs to recover in isolation for 24 h before returning them to a communal tank (see Note 5).
5. Place the freshly harvested ovaries in a glass Petri dish filled with OR2 buffer.
6. To remove the vascular follicle layer that surrounds the oocytes prior to injections, incubate ovaries in OR2 supplemented with 5 % Collagenase, 1 % Trypsin inhibitor and 1 % BSA for 2 h on a rocking platform (see Note 6).
7. Decant collagenase solution and wash oocytes several times in OR2 until the final solution is no longer turbid.
8. Wash twice with ND96.
9. Manually sort out healthy looking stage VI oocytes. Allow cells to recover for 3–6 h in ND96 buffer prior to microinjection (see Note 7).

3.3. Oocyte Microinjection

1. Pull glass injection needles with apertures of 15 μm or greater (see Note 8).
2. Centrifuge the concentrated protein sample for 10 min at 20,000 × *g* in a tabletop centrifuge at 4 °C to pellet any particular matter or precipitate that might otherwise clog the injection needle (see Note 9).

3. Mount the glass needle on the pneumatic injection device and calibrate by dispensing individual drops of protein sample in mineral oil. Measure the drop diameter under a microscope using the built-in micrometer scale. Choose injection-time and -pressure settings that yield injection volumes of 50 nL, or less (see Note 10).
4. For microinjections, seed prepared oocytes onto injection grids. Align cells with identical orientations in order to allow for rapid injections and comparable settings for needle penetration and sample deposition. These manipulations are carried out under a dissecting microscope.
5. Penetrate the immobilized oocytes at the equatorial plane separating the animal and the vegetal hemisphere of the cell with the injection needle. After sample deposition, withdraw the needle with equal precision and care.
6. Upon completion of oocyte injections, inspect all manipulated cells for the degree of incision and sort out any obviously damaged cells. Allow cells to recover for a minimum of 3 h (see Note 11).
7. Alternatively, oocyte manipulations can be carried with fully automated injection devices (see Note 12).
8. Following injections, transfer the oocytes to a large glass Petri dish. Wash cells thoroughly five times with excess volumes of ND96 at 45 min intervals and allow them to recover for at least 3 h.

3.4. Loading the NMR Tube

1. Transfer the injected oocytes to ND96 buffer containing 10 % D_2O.
2. Fill a Shigemi™ NMR tube with ND96/D_2O buffer (see Note 13). Carefully collect the oocytes with a pipette and add them individually to the tube, allowing them to sediment by gravity. Occasionally swirl the tube to ensure optimal settling and packing.
3. Count the number of oocytes per NMR sample and note the resulting volume of the specimen, as this will enable the accurate correlation of the effective NMR concentration (C_{NMR}) to the intracellular concentration (C_{Cell}) of the injected protein (see Note 14).
4. Proceed to the NMR spectrometer for in-cell NMR recordings (see Note 15).
5. Protein leakage from *Xenopus* oocytes should be assessed before-, and after in-cell NMR experiments. Carefully decant oocyte buffer and check for the presence of injected protein in the supernatant by Western blotting (12).

4. Notes

1. The concentration of IPTG, the induction temperature, and the duration of the induction can all be varied to find optimal conditions for maximal, soluble protein expression. The volume of cells required depends entirely on the protein yield; a single *Xenopus* oocyte in-cell NMR sample will typically require in excess of 2.5×10^{-8} mol of pure protein (~0.5–1 mM). Detailed protocols for recombinant protein expression and purification (13), as well as for stable-isotope labeling for NMR purposes (14), are available elsewhere.
2. Large affinity or fluorescent tags, such as GST, MBP, or GFP, are not recommended unless a specific protease cleavage site is introduced enabling the subsequent removal of the tag. Commonly used proteases include Factor X and TEV. Small peptide tags, especially hexahistidine, appear relatively innocuous in *Xenopus* oocytes, but will yield NMR resonances similar to those of IDPs and may obscure some chemical shifts of interest. Choose the final protein buffer considering the physiology of the oocytes and the need to keep the protein soluble. A good starting point is 50 mM sucrose, 150 mM NaCl, 25 mM HEPES, pH 7.5, 1 mM DTT.
3. Remember that subsequent injection steps will dilute the pure protein by a factor of ~20 upon oocyte delivery. This number is based on the notion that 50 nL of protein are injected into a cell volume of ~1 μL. Thus, if ~50 μM are envisaged as an arbitrary target for the effective concentration of the in-cell NMR sample (C_{NMR}), the oocyte experiment will require a starting protein concentration of at least 1 mM.
4. Alternatively, oocytes can be obtained without hormone priming. This is especially suitable during northern hemisphere winter months (*X. laevis* is an amphibian from South Africa and its intrinsic "seasonal" clock suggests a favorable summer climate when in fact winter prevails in the northern hemisphere). Oocyte quality may vary greatly from frog to frog. It is therefore suggested to hormone prime a couple of individuals in order to only select the best oocytes. On average, the number of healthy stage VI oocytes that can be obtained from a single frog, usually several hundred, suffice for multiple in-cell NMR experiments.
5. Oocytes are essentially prepared as described in ref. 15. This protocol represents the most up-to-date version of classical oocyte preparation routines (4). Frogs can also be sacrificed after oocyte removal, if laboratory regulations permit to do so.
6. Alternatively, the follicle layer can be removed by manually peeling it off under a dissecting microscope (16). This procedure

requires considerable practice and is quite time-consuming. The advantages over treating with collagenase are that follicle removal is complete, the oocytes are typically healthier, and components of the extracellular membrane have not undergone as harsh a treatment (which might not be critical for in-cell NMR measurements of intracellular proteins).

7. Stage VI oocytes are easily identified as the largest oocytes in the population, and they are typically healthy when the darkly pigmented hemisphere (the animal pole) appears rich and uniformly colored. About 200 oocytes are needed for a single in-cell NMR experiment. ND96 buffer can be substituted with MBS buffer, without calcium (88 mM NaCl, 1 mM KCl, 1 mM $MgSO_4$, 2.5 mM $NaHCO_3$, 5 mM HEPES, pH 7.55) at any stage of the oocyte preparation.
8. Concentrated protein solutions are typically more viscous than RNA that is most often injected into *Xenopus* oocytes. Therefore, the aperture of the needle needs to be wide enough to allow the protein solution to pass through while also small enough to minimize the trauma inflicted upon the oocytes. The aperture diameter should be determined empirically for each protein. Alternatively, pre-pulled needles can be obtained from commercial sources and have been found to consistently yield highly reproducible results, especially when used in combination with robotic injection devices (11, 17).
9. Some IDPs are aggregation prone and the highly concentrated injection solution may result in the formation of insoluble precipitates. Sedimentation of those species by an additional centrifugation step alleviates clogging problems. This step is particularly important when working with IDPs.
10. Most dissecting microscopes contain built in micrometer scales, visible through the ocular, which are used to determine the drop volume by employing the arithmetic diameter–volume relation assuming a perfect sphere shape (18).
11. Sakai et al. have reported the coinjection of an inert fluoroscopic dye in order to visually inspect for sample leakage (19). Possible dye binding to the protein of interest should first be ruled out by in vitro NMR measurements of a dye–protein mixture.
12. These systems can be employed to perform large-scale manipulation routines within a minimal amount of time (<2 min per 96-well plate). Mount pre-pulled injection needles (#38GC100TF-10, nozzle aperture ~15 μm, Multi Channel Systems) on a "standard" pneumatic oocyte injection device for sample loading through the needle tip and conventional drop-volume calibration in mineral oil. We found that this approach was superior to "back-loading" via the larger needle opening at

the mounting end, as the formation of trapped air bubbles is consistently avoided. Transfer the injection needle containing the labeled protein sample onto the injection arm of the robotic injection device and lock 96-well plates, containing one oocyte cell per well, onto the injection platform. Align the injection needle and 96-well plate manually according to the manufacturer's instructions. Automated injection procedures are typically carried out with settings of 0.1 bar holding pressure, 0.7 bar injection pressure, 200 ms injection time, and 500 μm injection depth, which corresponds to calibrated sample volumes of 50 nL per oocyte/injection (STD ±10 % or ±5 nL).

13. Bodart et al. have reported the usage of 20 % Ficoll in the final in-cell NMR buffer (9). The higher overall density of this solution significantly enhances the time span over which oocytes maintain their intact morphology. This appears to be due to reduced packing damage. Do not insert the Shigemi™ plunger, nor remove the excess buffer from the NMR tube for the in-cell experiment.

14. The effective NMR concentration (C_{NMR}) of the final in-cell NMR sample will be determined by the intracellular concentration of the "delivered" protein (C_{Cell}), the number of cells in the NMR sample (N_{Cell}, typically 200), their cell volume (V_{Cell}, 1 μL on average), the final NMR sample volume (V_{NMR}, typically 250 μL), and the corresponding volume dilution factor, VDF ($V_{NMR}/V_{Cell} \times N_{Cell}$). For *Xenopus* oocytes the VDF is usually 1.25. According to $C_{NMR} \times V_{NMR} = C_{Cell} \times (V_{Cell} \times N_{Cell})$ the effective NMR concentration can be calculated as $C_{NMR} = C_{Cell}/\mathrm{VDF}$. For example, in order to obtain a spectrum with an effective NMR concentration (C_{NMR}) of 10 μM of isotope labeled protein in 250 μL of NMR sample volume (V_{NMR}), for 200 cells (N_{Cell}) with an average cell volume (V_{Cell}) of 1 μL a total intracellular protein concentration (C_{Cell}) of 12.5 μM of isotope-labeled IDP must be reached. Given the dilution factor upon oocyte injection (50 nL injection volume into 1 μL of cell volume, i.e., ~20) the protein concentration in the injection needle has to be 250 μM.

15. Oocyte in-cell NMR samples are inherently inhomogeneous. For this reason, any attempts to shim the sample will be unsuccessful and should be omitted altogether. NMR pulse calibrations are best set up by fast one-dimensional heteronuclear correlation experiments varying the individual pulse lengths in small increments. Due to the high viscosity of the intracellular environment, pulses are typically longer than for pure in vitro samples (although this increase in pulse lengths is typically less drastic for IDPs with high internal mobility).

References

1. Ito Y, Selenko P (2010) Cellular structural biology. Curr Opin Struct Biol 20:640–648
2. Uversky VN, Oldfield CJ, Midic U, Xie H, Xue B, Vucetic S, Iakoucheva LM, Obradovic Z, Dunker AK (2009) Unfoldomics of human diseases: linking protein intrinsic disorder with diseases. BMC Genomics 10 Suppl 1: S7
3. Selenko P, Frueh DP, Elsaesser SJ, Haas W, Gygi SP, Wagner G (2008) In situ observation of protein phosphorylation by high-resolution NMR spectroscopy. Nat Struct Mol Biol 15:321–329
4. Murray AW (1991) *Xenopus laevis*: practical uses in cell and molecular biology. Methods Cell Biol 36:1–718
5. Dedmon MM, Patel CN, Young GB, Pielak GJ (2002) FlgM gains structure in living cells. Proc Natl Acad Sci USA 99:12681–12684
6. Crane R, Ruderman J (2006) Using *Xenopus* oocytes to study signal transduction. Methods Mol Biol 322:435–445
7. Sakai T, Tochio H, Tenno T, Ito Y, Kokubo T, Hiroaki H, Shirakawa M (2006) In-cell NMR spectroscopy of proteins inside *Xenopus laevis* oocytes. J Biomol NMR 36:179–188
8. Selenko P, Serber Z, Gadea B, Ruderman J, Wagner G (2006) Quantitative NMR analysis of the protein G B1 domain in *Xenopus laevis* egg extracts and intact oocytes. Proc Natl Acad Sci USA 103:11904–11909
9. Bodart JF, Wieruszeski JM, Amniai L, Leroy A, Landrieu I, Rousseau-Lescuyer A, Vilain JP, Lippens G (2008) NMR observation of Tau in *Xenopus* oocytes. J Magn Reson 192:252–257
10. Hansel R, Foldynova-Trantirkova S, Lohr F, Buck J, Bongartz E, Bamberg E, Schwalbe H, Dotsch V, Trantirek L (2009) Evaluation of parameters critical for observing nucleic acids inside living *Xenopus laevis* oocytes by in-cell NMR spectroscopy. J Am Chem Soc 131:15761–15768
11. Serber Z, Selenko P, Hansel R, Reckel S, Lohr F, Ferrell JE Jr, Wagner G, Dotsch V (2006) Investigating macromolecules inside cultured and injected cells by in-cell NMR spectroscopy. Nat Protoc 1:2701–2709
12. Lee C (2007) Western blotting. Methods Mol Biol 362:391–399
13. Hewitt L, McDonnell JM (2004) Screening and optimizing protein production in *E. coli*. Methods Mol Biol 278:1–16
14. Serber Z, Corsini L, Durst F, Dotsch V (2005) In-cell NMR spectroscopy. Methods Enzymol 394:17–41
15. Sive HL, Grainger RM, Harland RM (2010) Isolation of *Xenopus* oocytes. Cold Spring Harb Protoc. pdb prot5534
16. Sive HL, Grainger RM, Harland RM (2010) Defolliculation of *Xenopus* oocytes. Cold Spring Harb Protoc. pdb prot5535
17. Schnizler K, Kuster M, Methfessel C, Fejtl M (2003) The roboocyte: automated cDNA/mRNA injection and subsequent TEVC recording on *Xenopus* oocytes in 96-well microtiter plates. Receptors Channels 9:41–48
18. Sive HL, Grainger RM, Harland RM (2010) Calibration of the injection volume for microinjection of *Xenopus* oocytes and embryos. Cold Spring Harb Protoc. pdb prot5537
19. Sakai T, Tochio H, Inomata K, Sasaki Y, Tenno T, Tanaka T, Kokubo T, Hiroaki H, Shirakawa M (2007) Fluoroscopic assessment of protein leakage during *Xenopus* oocytes in-cell NMR experiment by co-injected EGFP. Anal Biochem 371:247–249