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Xenopus laevis Oocytes Preparation for in-Cell EPR Spectroscopy

Laura John and Malte Drescher*

Department of Chemistry and Konstanz Research School Chemical Biology, University of Konstanz, Konstanz, Germany
*For correspondence: malte.drescher@uni-konstanz.de

[Abstract] One of the most exciting perspectives for studying bio-macromolecules comes from the emerging field of in-cell spectroscopy, which enables to determine the structure and dynamics of bio-macromolecules in the cell. In-cell electron paramagnetic resonance (EPR) spectroscopy in combination with micro-injection of bio-macromolecules into *Xenopus laevis* oocytes is ideally suited for this purpose. *Xenopus laevis* oocytes are a commonly used eukaryotic cell model in different fields of biology, such as cell- and development-biology. For in-cell EPR, the bio-macromolecules of interest are microinjected into the *Xenopus laevis* oocytes upon site-directed spin labeling. The sample solution is filled into a thin glass capillary by means of Nanoliter Injector and after that microinjected into the dark animal part of the *Xenopus laevis* oocytes by puncturing the membrane cautiously. Afterwards, three or five microinjected *Xenopus laevis* oocytes, depending on the kind of the final in-cell EPR experiment, are loaded into a Q-band EPR sample tube followed by optional shock-freezing (for experiment in frozen solution) and measurement (either at cryogenic or physiological temperatures) after the desired incubation time. The incubation time is limited due to cytotoxic effects of the microinjected samples and the stability of the paramagnetic spin label in the reducing cellular environment. Both aspects are quantified by monitoring cell morphology and reduction kinetics.
Keywords: *Xenopus laevis* oocytes, in-Cell EPR, in-Cell spectroscopy, Site-directed spin labeling, Microinjection, *in vivo* structure determination, Dynamics of biomacromolecules

[Background] Electron paramagnetic resonance (EPR) spectroscopy is the method of choice for characterization of paramagnetic systems (Atherton, 1993; Gerson *et al.*, 1994; Jeschke and Schweiger, 2001). Diamagnetic bio-macromolecules can be made accessible for EPR spectroscopy by site-directed spin labeling (SDSL), commonly using nitroxides as spin labels (Hubbell and Altenbach, 1994; Feix and Klug, 2002; Likhtenshtein *et al.*, 2008; Berliner and Reuben, 2012). The combination of SDSL with in-cell EPR spectroscopy is a powerful tool to gain information about structure and dynamics of bio-macromolecules such as proteins or nucleotides in their natural environment (Azarkh *et al*., 2013; Martorana *et al*., 2014; Qi *et al*., 2014; Cattani *et al*., 2017). The most common experimental procedure for the fledging technique of in-cell EPR is based on the microinjection of the target molecules into oocytes from the African frog *Xenopus laevis*, which are a widely used eukaryotic cell model (Kay, 1991; Barnard *et al*., 1982; Mishina *et al*., 1984; Dawid and Sargent, 1988; Richter, 1999).

The advantages of *Xenopus laevis* oocytes for in-cell EPR are the large size of approximately 1 mm in diameter (approximately 1 µl cell volume), the resulting easy handling and the fact that only three or

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five of them are required for an in-cell EPR sample (Qi *et al*., 2014; Cattani *et al*., 2017). Consequently, bio-macromolecules can be introduced relatively easily in the quantity required for EPR measurements into the *Xenopus laevis* oocyte by microinjection. Hence, there have been numerous intracellular distance measurements of spin labelled DNA and proteins performed by double electron-electron resonance (DEER) measurements after microinjection into *Xenopus laevis* oocytes (Igarashi *et al*., 2010; Azarkh *et al*., 2011; Krstic *et al*., 2011; Azarkh *et al*., 2013; Martorana *et al*., 2014; Wojciechowski *et al*., 2015; Cattani *et al*., 2017).

Materials and Reagents

1. Glass capillaries (3.5 inch length, Drummond Scientific, catalog number: 3-000-203-G/X)
2. Single-use syringe (Sigma-Aldrich, catalog number: Z230723)
3. Parafilm (Sigma-Aldrich, catalog number: P7793-1EA)
4. Petri dish, size 60 x 15 mm (Corning, catalog number: 430166)
5. Razor blade (Plano, catalog number: T5016)
6. Pasteur capillary pipette (150 mm, WU Mainz)
7. Brand pipette controller micro-classic (BRAND, catalog number: 25900)
8. Q-band sample tubes (quartz glass, 1 mm i.d., Bruker, catalog number: ER 221TUB-Q10)
9. Hamilton syringe (Hamilton, catalog number: 80500)
10. Petri dish, size 35 x 10 mm (Corning, catalog number: 430165)
11. Capillary tube sealing compound (Cha-seal, DWK Life Sciences, Kimble, catalog number: 43510)
12. *Xenopus laevis* oocytes on stage V/VI in MBS buffer (Ecocyte Bioscience, ecocyte-us.com/products/xenopus-oocyte-delivery-service/)
13. Modified Barth's Saline (MBS) buffer (1x) (Ecocyte Bioscience) (88 mM NaCl, 1 mM KCl, 1 mM $MgSO_4$, 5 mM HEPES, 2.5 mM $NaHCO_3$, 0.7 mM $CaCl_2$)
14. Mineral oil (Sigma-Aldrich, catalog number: M5904)
15. Liquid nitrogen
16. 3-Maleimido-PROXYL (3-Maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy) (Sigma-Aldrich, catalog number: 253375)

Equipment

1. Flaming/Brown Micropipette Puller (Sutter Instrument, model: P-97)
2. Nanoject II Auto-Nanoliter Injector (Drummond Scientific, catalog number: 3-000-205A)
3. Micromanipulator MM33 (Drummond Scientific, catalog number: 3-000-024-R) with Support Base (Drummond Scientific, catalog number: 3-000-025-SB)
4. Binocular microscope (ZEISS, model: Stemi 2000-C, attended with an AxiaCam ERc 5s camera (ZEISS, model: AxiaCam ERc 5s))

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5. Home-built polytetrafluoroethylene holder
6. Dewar for liquid nitrogen (KGW-Isotherm, catalog number: 1021)
7. -80 °C freezer

Procedure

A. Preparation of the injector glass capillary

1. The desired thin top of the glass capillary is formed with the Flaming/Brown Micropipette Puller. The program parameters are chosen as follows: Pressure Setting (P) = 500, Heat = 558, Pull = 100, Velocity (VEL) = 120 and Time = 100 msec (agrees with 200 units).
2. The exact position of the Nanoliter Injector can be adjusted with the micromanipulator. At the front of the Nanoliter Injector are a screw clip and a stamp, the position of the latter is controlled by the control computer (see Figure 1).

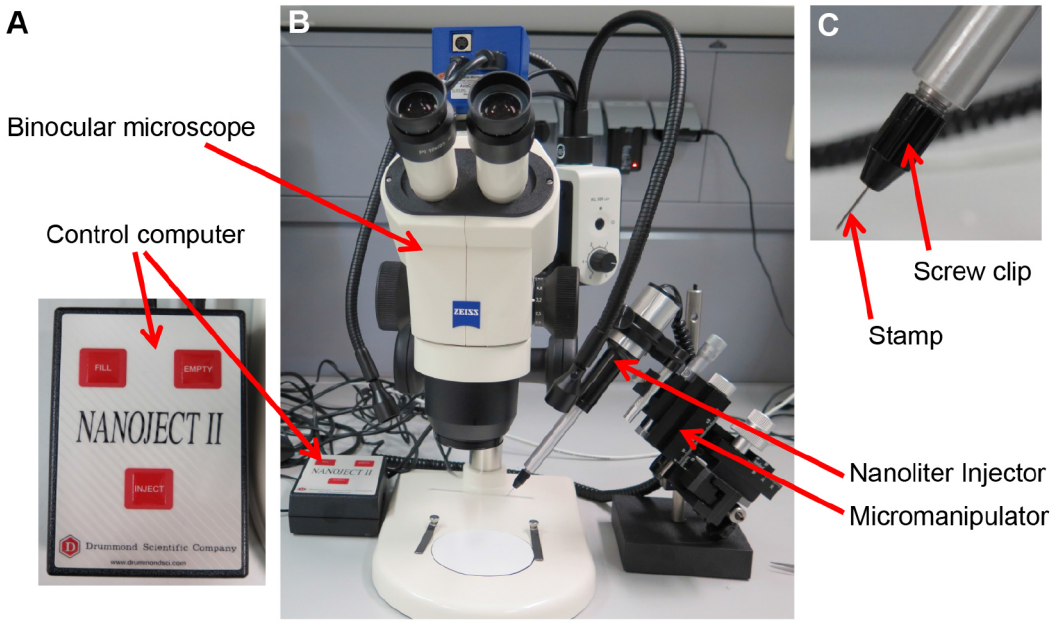

Figure 1. Assembly of the microinjection equipment. A. Magnified view of the control computer; B. Binocular microscope and microinjection equipment; C. Magnified view of the top of the Nanoliter Injector.

3. To avoid air pockets between the stamp and the injection solution, the sharpened glass capillary is filled up with mineral oil using a single-use syringe. Afterwards, the filled glass capillary is clamped over the stamp into the screw clip of the Nanoliter Injector. Exiting mineral oil is wiped away with a tissue.
4. For the opening of the clamped glass capillary, Parafilm is stretched over the back of a Petri dish (size 60 x 15 mm). A small mark is pressed into the Parafilm at the margin of the Petri dish. The glass capillary is slowly moved towards the mark on the Petri dish with the micromanipulator under observation through the binocular microscope. When the glass capillary starts touching

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the Parafilm, it is put down horizontally on the Parafilm by simultaneously lowering the capillary with the micromanipulator and moving the Petri dish away from the latter. The laid down, forward piece of the glass capillary (around 2.5 mm) is cut carefully with a razor blade.

5. Subsequently, the mineral oil is pressed out of the opened glass capillary up to around 2 cm with the stamp of the Nanoliter Injector. This run-off can take up to 15 min.
6. The top of the prepared glass capillary is dipped into a drop of the sample solution (2-4 µl depending on the number of *Xenopus laevis* oocytes to be microinjected). The sample solution is soaked up into the capillary by using the Nanoliter Injector (see Figure 2 for prepared injector glass capillary).

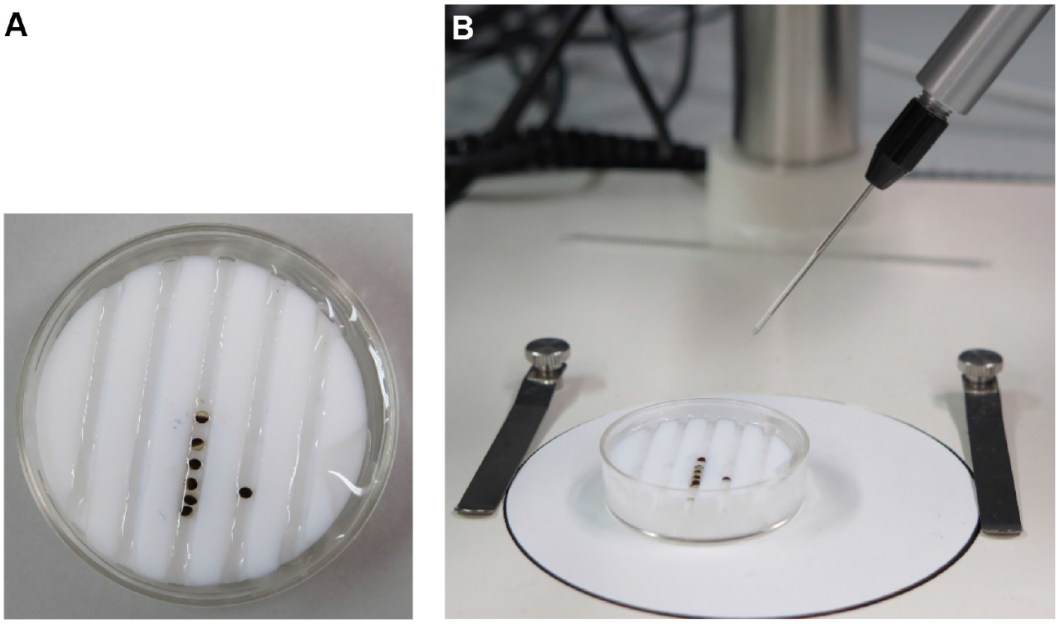

Figure 2. Ready prepared *Xenopus laevis* oocytes and glass capillary. A. Magnified view of the self-made polytetrafluoroethylene holder with *Xenopus laevis* oocytes in MBS buffer. B. Prepared injector glass capillary clamped in the Nanoliter Injector with the self-made polytetrafluoroethylene holder.

B. Microinjection of the *Xenopus laevis* oocytes

1. The *Xenopus laevis* oocytes at stage V/VI in MBS buffer can be ordered from Ecocyte Bioscience and arrive cooled the next day. They are kept in MBS buffer at 18 °C (room temperature is set to 18 °C) and used for the sample preparation not later than the day of delivery.
2. For the microinjection, 6-7 *Xenopus laevis* oocytes are prepared with a Pasteur capillary pipette on a self-made polytetrafluoroethylene (PTFE) holder (produced by PTFE milling, size 35 x 10 mm, with 1 mm wide and 2 mm high grooves at a distance of 4 mm) in MBS buffer, in which they are shipped, and visually inspected for their state with the binocular microscope (see Figure 2). *Xenopus laevis* oocytes with initial signs of apoptosis, such as flabby membrane or light

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discolorations in the dark animal hemisphere of the *Xenopus laevis* oocyte, are rejected (for comparison of healthy and damaged *Xenopus laevis* oocytes see Figure 3).

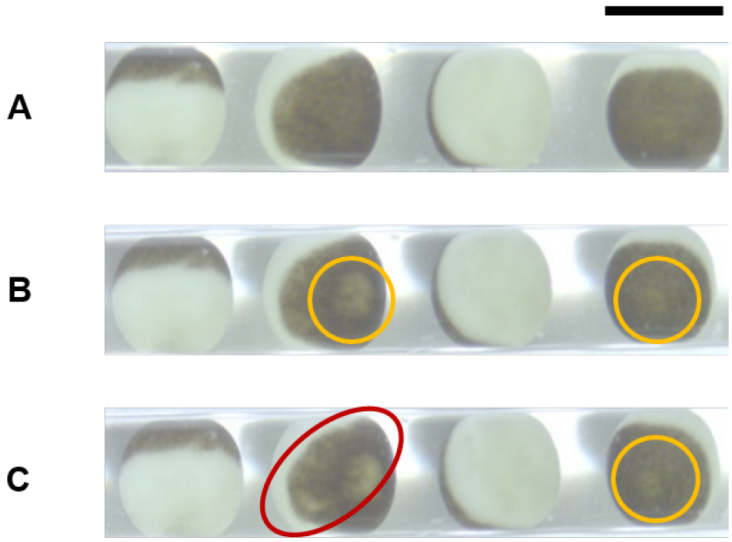

Figure 3. Micrographs of *Xenopus laevis* oocytes. A. Healthy *Xenopus laevis* oocytes without signs of apoptosis; B. and C. Light discolorations in the dark animal hemisphere of the *Xenopus laevis* oocytes as an initial sign of apoptosis are tagged by orange circles. C. Progressed apoptosis in the form of flabby membrane and cell deformation is tagged by a red ellipse. Scale bar = 1 mm.

3. The membrane of the *Xenopus laevis* oocytes is punctured carefully in the dark animal hemisphere, near the parting line, which separates the animal hemisphere from the light vegetal hemisphere of the *Xenopus laevis* oocyte. The penetrating glass capillary is moved in the direction of the parting line so that the glass capillary points at the parting line from diagonally above. The injection of the sample solution (typically 50 nl) is carried out in this position by the Nanoliter Injector so that the nucleus in the animal hemisphere will not be injected (see Figure 4).

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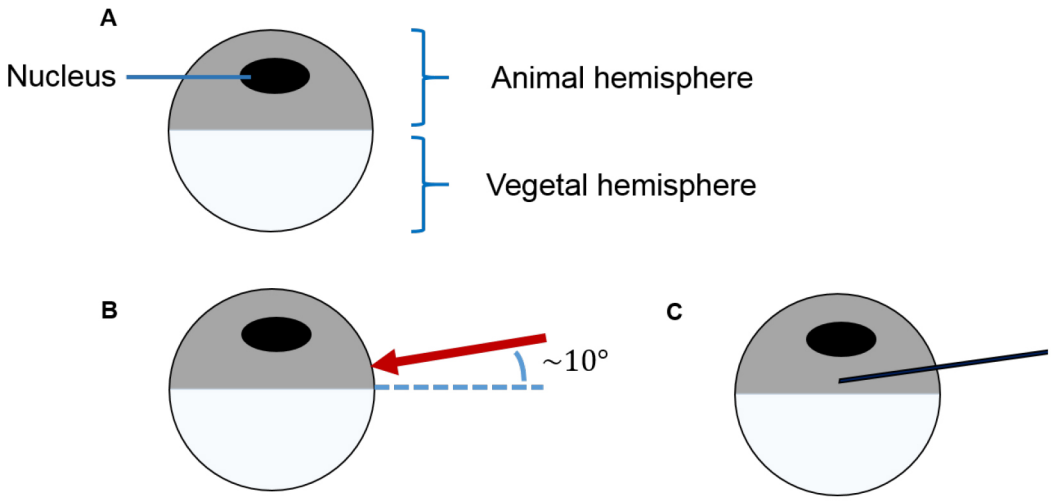

Figure 4. Process flow of the microinjection. A. Diagram of a *Xenopus laevis* oocyte; B. For the microinjection, the glass capillary penetrates the *Xenopus laevis* oocyte in the animal hemisphere, very close to the vegetal hemisphere, at an approximately 10° angle, tagged by a red arrow. C. Position of the glass capillary (shown in dark blue) during the injection.

4. The glass capillary is removed by carefully pulling it off. Injured *Xenopus laevis* oocytes are thrown away, while successfully injected *Xenopus laevis* oocytes are washed carefully with MBS buffer.

C. Collecting in an EPR sample tube

1. Upon microinjection, three (for pulsed Q-band EPR measurements at ~34 GHz) or five (for X-band continuous wave [cw] EPR measurements at ~9.5 GHz) *Xenopus laevis* oocytes are carefully transferred into a Q-band EPR sample tube. For this, a slight negative pressure is built up with a pipette controller on one end of the sample tube, which is moistened with MBS buffer.
2. Initially a small volume of MBS buffer is collected, followed by the *Xenopus laevis* oocytes and again a small volume of MBS buffer. Care should be taken to ensure that there is no space between the *Xenopus laevis* oocytes within the sample tube. Furthermore, it must be ensured by visual inspection using the binocular microscope that the cell membrane of the *Xenopus laevis* oocytes is not damaged through the collection and that no air pockets are in the sample tube. Otherwise, the sample is unusable. This visual inspection must be performed again after the desired incubation time of the *Xenopus laevis* oocytes at 18 °C.
3. The incubation time has to be selected in accordance with the morphological stability of the *Xenopus laevis* oocytes upon microinjection and the stability of the paramagnetic spin label in the reducing environment of the *Xenopus laevis* oocyte. Both are determined prior to the final in-cell EPR experiment (see Data analysis).
4. After the incubation time at 18 °C, the supernatant MBS buffer in the sample tube is removed using a Hamilton syringe in such a way that the MBS buffer just covers the edge of the outer

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Xenopus laevis oocytes, maximum 1-2 mm distance from the edge of the *Xenopus laevis* oocytes (finished prepared sample tube see Figure 5).

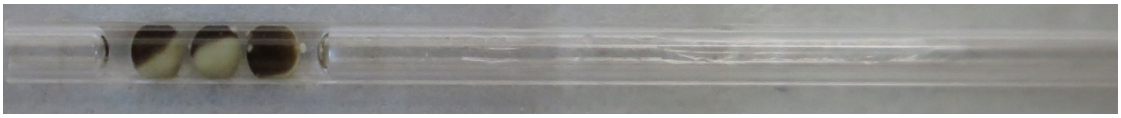

Figure 5. Prepared Q-band sample tube with three *Xenopus laevis* oocytes in MBS buffer immediately before shock-freezing for pulsed Q-band EPR measurements

5. For pulsed Q-band EPR measurements (~34 GHz) the sample is subsequently shock-frozen in liquid nitrogen and stored in the freezer at -80 °C until measuring without thawing. In contrast, for X-band cw-EPR measurements (~9.5 GHz) at 20 °C the sample tube is sealed with the capillary tube sealing compound at the top to guarantee a fixed sample position within the tube. Furthermore, X-band cw-EPR measurements must be performed immediately after sample preparation because the stability of the paramagnetic spin label inside the cell and the morphological stability of the microinjected *Xenopus laevis* oocytes might be time-limited at 20 °C (see Data analysis).

Data analysis

1. Investigating cytotoxic effects of injected samples: The morphological stability of the *Xenopus laevis* oocytes has to be checked upon microinjection of the paramagnetic sample solution to ensure that after the chosen incubation time all cells in the sample are still intact. This can be done with a morphological study over several hours, as suggested by Groß *et al*. (Qi *et al*., 2014; Wojciechowski *et al*., 2015).
2. Investigating stability of paramagnetic spin label: The stability of the spin labels in the reducing environment of the *Xenopus laevis* oocytes is tested by measuring the X-band cw-EPR signal of the labeled macromolecules microinjected into *Xenopus laevis* oocytes at 18 °C. The decay of the signal intensity corresponds to a reduction of the paramagnetic spin labels under the applied conditions. There are different half-times of the signal decay for different spin labels at 18 °C in *Xenopus laevis* oocytes (Qi *et al*., 2014; Karthikeyan *et al*., 2017).
   For spin labels featuring sufficient stability, incubation times around 60 min at 18 °C are typically chosen (Qi *et al*., 2014; Wojciechowski *et al*., 2015). Using 3-maleimido-PROXYL (3-Maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy) spin labels, typical incubation time at 18 °C is in the range of 15 min (Cattani *et al*., 2017).
3. Endogenous paramagnetic species: *Xenopus laevis* oocytes contain endogenous paramagnetic Mn(II) species, which are also represented in EPR spectra (Qi *et al*., 2014). Thus, untreated *Xenopus laevis* oocytes are measured for background correction (Qi *et al*., 2014; Cattani *et al*., 2017).

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4. Spatial expansion of the injected volume by diffusion: In order to determine the effect of translational diffusion of the target molecules within the *Xenopus laevis* oocyte upon microinjection, the local concentration can be determined by double electron-electron resonance (DEER) measurements (Jeschke *et al*., 2006; Cattani *et al*., 2017).

Notes

1. Compressible air pockets in the glass capillary would lead to a wrong injection volume and injection velocity.
2. It is important to neither cut too much nor too little of the glass capillary when you open it with the razor blade. If too much is cut off, the diameter of the glass capillary will be too big for microinjection without extensively damaging the *Xenopus laevis* oocytes. If too little is cut off, the stability of the class capillary will not suffice to penetrate *Xenopus laevis* oocytes.
3. Ensure that the *Xenopus laevis* oocytes are always covered with MBS buffer solution.
4. For a quick succession of the microinjections, it is useful to align the *Xenopus laevis* oocytes in the polytetrafluoroethylene holder in the same way by carefully turning them with the glass capillary clamped in the Nanoliter Injector.
5. The loading of the *Xenopus laevis* oocytes into the Q-band sample tube is facilitated by a Parafilm-strip on a Petri dish (size 35 x 10 mm) forming an elevation in the middle of the dish (see Figure 6). The *Xenopus laevis* oocytes are given with a Pasteur pipette into a drop of MBS buffer onto the Petri dish directly next to the elevation modeled out of Parafilm. By slightly pushing the *Xenopus laevis* oocyte with the Q-band sample tube against the Parafilm-elevation, the *Xenopus laevis* oocytes can be brought into a good position for the collection of the *Xenopus laevis* oocytes into the sample tube without space between each of them.

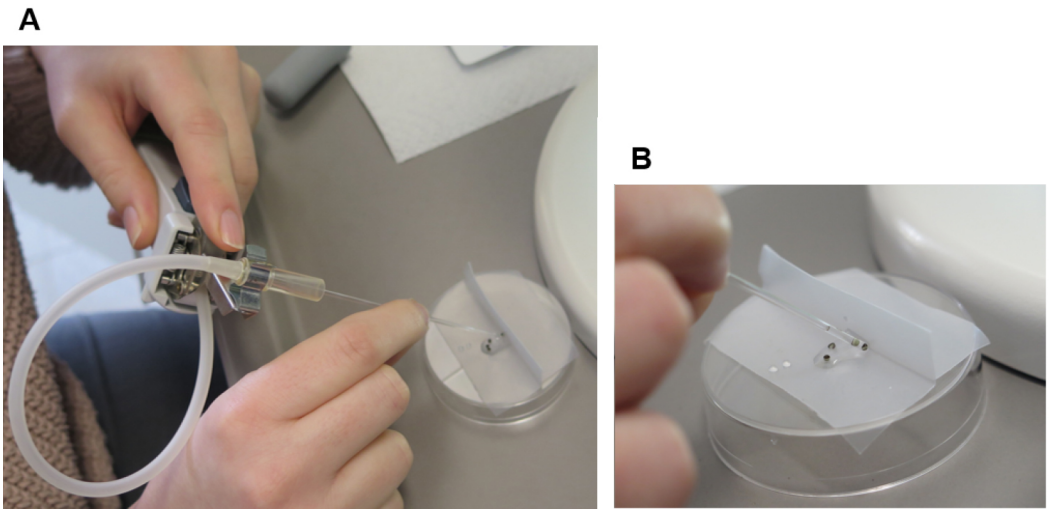

Figure 6. Assembly for an easy collection of the *Xenopus laevis* oocytes into a Q-band sample tube. A. *Xenopus laevis* oocytes positioned in a drop of MBS buffer on a Petri dish directly next to an elevation made out of Parafilm can be easily collected in a Q-band sample

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tube by means of a pipette controller. B. Magnified view of the positioning and collection of the *Xenopus laevis* oocytes.

Acknowledgments

The protocol described has been developed and improved by different members of the Research Group of Prof. Dr. Malte Drescher and was based on previous protocols of Dr. Andreas Groß (Qi *et al*., 2014; Wojciechowski *et al*., 2015) and Dr. Julia Cattani (Cattani *et al*., 2017). We thank Juliane Stehle and Anna Bieber for the pictures in Figure 6. Support by the Deutsche Forschungsgemeinschaft within the priority programme SPP 1601 is gratefully acknowledged. The authors declare no conflicts of interest or competing interests.

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