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Tips and tricks for preparing lampbrush chromosome spreads from *Xenopus tropicalis* oocytes

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ABSTRACT

Due to their large size and fine organization, lampbrush chromosomes (LBCs) of amphibian oocytes have been for decades one of the favorite tools of biologists for the analysis of transcriptional and post-transcriptional processes at the cytological level. The emergence of the diploid *Xenopus tropicalis* amphibian as a model organism for vertebrate developmental genetics and the accumulation of sequence data made available by its recent genomic sequencing, strongly revive the interest of LBCs as a powerful tool to study genes expressed during oogenesis. We describe here a detailed protocol for preparing LBCs from *X. tropicalis* oocyte and give practical advice to encourage a large number of researchers to become familiar with these chromosomes.

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1. Introduction

Anyone who has observed a cytological preparation of amphibian lampbrush chromosomes (LBCs) with a phase-contrast microscope has immediately fallen under the spell of these giant chromosomes. LBCs are observed in the oocytes of vertebrates and invertebrates but they are particularly developed in amphibians [1]. They are not confined to the animal kingdom and lampbrush-type chromosomes have been described in the green alga, *Acetabularia mediterranea* [2] and in characean algae [3]. They were first described by Flemming [4] in the oocyte nucleus or germinal vesicle (GV) of the salamander *Ambystoma mexicanum* but the name "lampbrush" was given to them by Rückert [5] for their resemblance to oil-lamp brushes used in the 19th century. As shown in Fig. 1, the two homologues are associated in bivalents and several thousand pairs of lateral loops unfold along their axis, giving them a characteristic feathery aspect. Each loop is the site of intense transcriptional activity and the nascent RNA transcripts together with the bound proteins form the RNP fibrils. The main feature of LBCs is their dynamic architecture directly related to transcriptional and post-transcriptional processes. Any modification of these processes is reflected by concomitant morphological variations of their structure. This is why LBCs have been used for several decades as a powerful model for cytological and biochemical analyses of transcriptional processes (for a review see [6]). More recently, LBCs were also used as a tool for the dissection of complex chromosomal processes such as the cohesion of sister chromatids [7].

The recent sequencing of the diploid genome of *Xenopus tropicalis* makes it an attractive model organism for developmental genetics studies in amphibians. LBCs of *X. tropicalis* provide a valuable tool for studying the molecular organization and function of genomic sequences, which are expressed during oogenesis. *In situ* hybridization of specific probes to nascent transcripts of lateral loops consistently yields very strong signals because the probes bind to numerous closely packed RNA transcripts in these loops (Fig. 1). The localization of the hybridizing loops can be defined accurately using the available LBCs maps of the ten bivalents of the oocyte karyotype [8].

Duryee [9] was the first to demonstrate that LBCs can be hand-isolated from the living oocytes of a frog. In 1960s, Gall [10] and Callan and Lloyd [11] developed methods for preparing newt lampbrush chromosomes for observation by phase-contrast microscopy. Thereafter, this technique with some variations was applied successfully to the mapping of LBCs of different urodelan and anuran species (reviewed by Callan [1]), the latest of these studies to date being the establishment of working maps of *X. laevis* [12] and *X. tropicalis* LBCs [8].

LBCs are observed throughout oogenesis from the early diplotene stage, i.e. Dumont stage I [13] [14]. It is well known from studies of LBCs of different amphibians species that their size and the

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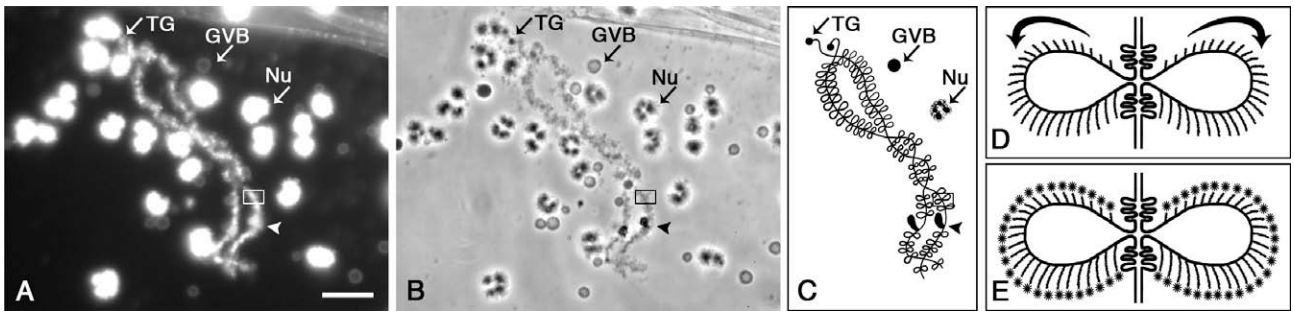

Fig. 1. A GV spread. Fluorescent (A) and corresponding phase contrast (B) micrographs of the content of a GV spread from paraformaldehyde-fixed and propidium iodine-stained preparation observed under a Leica microscope using a Leitz DMRB CCD camera. Bivalent VII, one of the 10 bivalents of the *X. tropicalis* karyotype, and numerous nucleoli (Nu) and GV bodies (GVB) are shown. This bivalent can be identified by the presence of lateral loops of fibrillar matrix (arrowhead) located near its right end and by terminal granules at its left extremity (TG) (for details, see [8]). (C–E) Schematic representations of the organization of LBCs. (C) Each bivalent is formed by two homologues remaining associated at the chiasmata. Each homologue display several hundred lateral loops which exhibit the same "standard type" structure while a few others exhibit distinctive structures considered as "landmarks" (arrowhead). (D) Schematic representation of one pair of lateral loops. The chromatid axes unfold in paired loops extending in opposite directions. The unfolded DNA is a site of intense transcriptional activity. The increasing size of the nascent transcripts indicates the progress of transcription (arrows). (E) Schematic representation of the enhancement of the *in situ* hybridization signal by the binding of specific probes (stars) to the closely packed RNA transcripts.

degree of development of their lateral loops are directly related to their transcriptional activity. LBCs reach their optimal size at Dumont stages III–IV when transcription is at its maximum. At Dumont stage VI, when transcription slows down, chromosomes become shorter and their lateral loops regress. Although LBCs are observed as early as Dumont stage I, LBCs spreads are usually performed with stages IV–VI oocytes. Preparation of satisfactory GV spreads from stages I to III oocytes is considered difficult because their GVs are too small to handle easily. This is the reason why Gall speaking of *X. laevis* stated that only the most intrepid lampbrushlogist would attempt a conventional GV spread from an oocyte with a diameter less than 0.5 mm [15]. *X. laevis* stage V oocytes which are 1 mm in diameter are considered to have an optimal size. *X. tropicalis* oocytes and their corresponding GVs have a smaller size than those of *X. laevis* but we found that this was not a limiting factor for preparing LBCs spreads including those from GVs of stage III oocytes. We describe here a detailed protocol, which allows good quality LBCs spreads to be obtained from *X. tropicalis* oocytes in a routine fashion.

2. A protocol for *X. tropicalis* lampbrush chromosomes spreads

The protocol we used for the construction of the working map of the 10 LBCs of *X. tropicalis* [8] was modified from that established for *X. laevis* LBCs by Callan et al. [12] and detailed further by Gall et al. [15]. A comparison of the main steps of these two protocols is provided in Table 1 and the different steps of our protocol for LBCs spreads of *X. tropicalis* are illustrated in Fig. 2. The composition of the working solutions and the description of the material necessary for preparing LBCs spreads are reported below (Appendix).

2.1. The different stages of oocyte development in the X. tropicalis ovary

On the basis of the classification criteria introduced by Dumont [13] for *X. laevis* and according to the size of the oocyte and pigment distribution, six stages of oocyte development can be distinguished in the *X. tropicalis* ovary (Fig. 3). *X. tropicalis* oocytes are smaller than those of *X. laevis* (Table 2). Stage I oocytes (50–100 μm in diameter) are transparent while those at stage II (200–250 μm in diameter) are of white color. Stage III oocytes (350–400 μm in diameter) appear uniformly grey. At stage IV (400–500 μm in diameter) the pigmented animal hemisphere and the yellowish vegetal hemisphere become clearly differentiated. Stage V oocytes (600–700 μm in diameter) can be distinguished from stage VI oocytes (700–800 μm in diameter) by their smaller size. The ovaries of hormonally- stimulated females also contain oocytes at the same stages of development, but the number of stage VI oocytes is dramatically decreased as shown in Fig. 3.

2.2. Detailed protocol

2.2.1. Preparation of females

Mature females are maintained unfed for two days before surgery. Hormonally stimulated females are injected with human Chorionic Gonadotropin (100 IU/100 μl) into the left or right lymph sac to induce ovulation, which occurs normally 12–24 h later. Ovary biopsy is performed within 2 days following ovulation.

2.2.2. Ovary biopsy

Females are anesthetized for 30–40 min by immersion into 0.1% MS222 (Amino-benzoic Acid Ethyl, Fluka) until they are completely immobile. Because the anesthetic solution is acidic (pH 4–5), it is important to rinse the animals with tap water before surgery. The females are placed on their back and a longitudinal 0.5 cm incision is made on the left or the right side in the posterior half of the trunk using a surgical blade. The use of a surgical blade is preferable to that of iridectomy scissors in order to make a sharp cut through the skin and the underneath muscular wall at the same time. Using forceps with rounded ends, a small piece of ovary is removed and placed into a Petri dish in MBS Buffer (modified Barth's solution) [16] or OR2 medium (oocyte Ringer's medium) [17] at room temperature (RT). The incision is closed with thread silk or cotton suture. Females are maintained under observation in a small aquarium in the laboratory for the next two days before

Table 1
Comparison of the main steps used to prepare LBCs from *X. tropicalis* and *X. laevis*.

	X. tropicalis	*X. laevis*
Oocyte stages used for LBCs spreads	III–VI	IV–V
Extraction of the GVs in the "Isolation Medium"	Embryo dish	Small petri dish
Recovery of the GV content	Directly in the dispersal chamber	Indirectly after transfer in small Petri dish of "Dispersal" medium
Duration and speed of centrifugation	10 min 300*g* + 30 min 3100*g*	60 min 3100–4800*g*

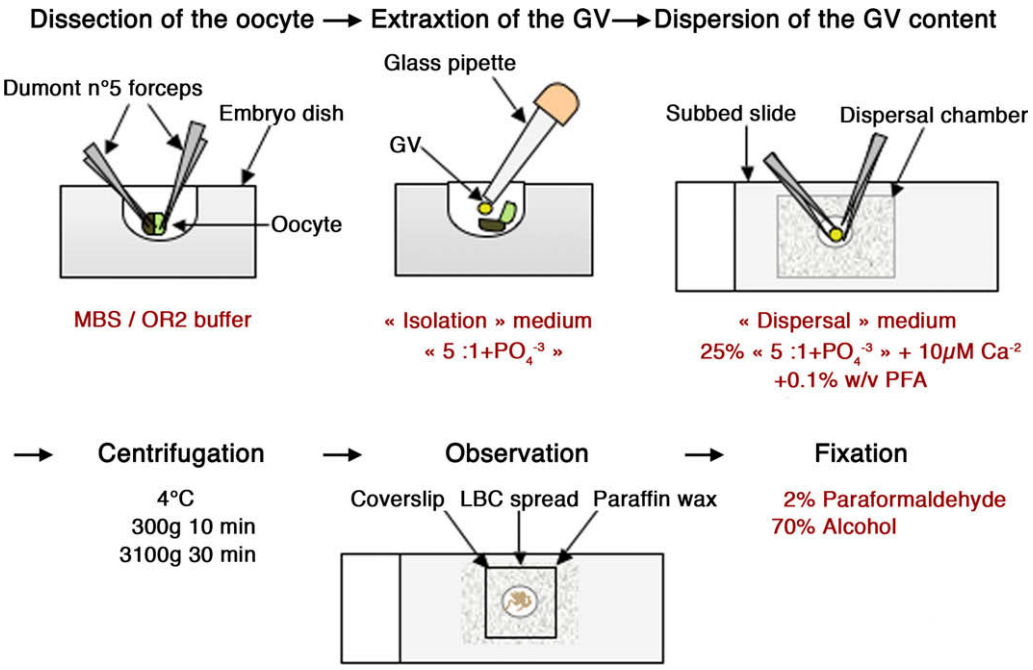

Fig. 2. Summary of the different steps in the preparation of LBCs spreads from *X. tropicalis* oocytes.

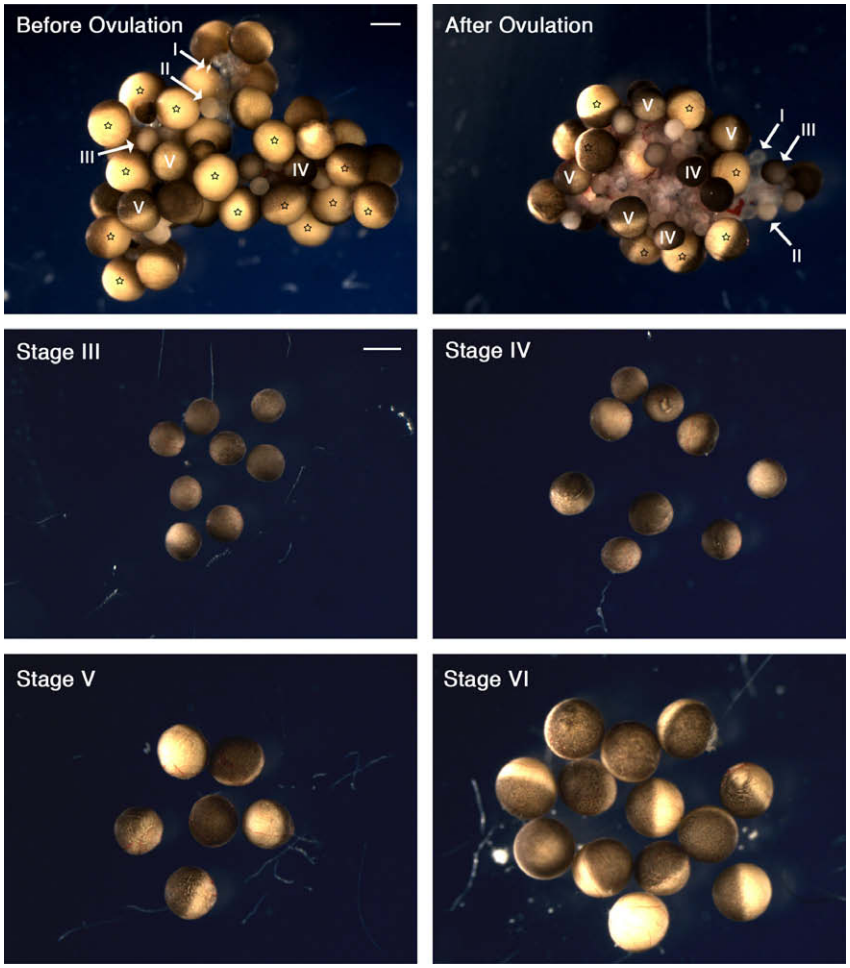

Fig. 3. The different stages of oocyte development in the *X. tropicalis* ovary. Top row: ovary fragments from the same *X. tropicalis* female before and after hormonal stimulation. Note that after ovulation, the number of stage VI oocyte (stars) is decreased. Lower panels: isolated oocytes at different stages of development. Scale bars represent 0.5 mm.

Table 2
Size of oocytes of *X. laevis* [13] and *X. tropicalis* at stages III–VI of development.

Stage	I	II	III	IV	V	VI
Size (μm) of *X. laevis* oocytes	50–300	300–450	450–600	600–1000	1000–1200	1200–1300
Size (μm) of *X. tropicalis* oocytes	≈250	≈350	350–400	400–500	500–600	600–700

returning to the main aquarium. The incision heals after a few days, but it is preferable to wait 3–4 weeks before operating again the same animal.

2.2.3. Oocytes sorting

From this step onwards, all operations are carried out under a binocular dissecting microscope. At lower magnification (0.8×), the oocytes are gently dissociated in the MBS medium using two pairs of forceps (Dumont, No. 5) and sorted according to their stage. Only stages III to VfI oocytes are retained (Fig. 3). They are rinsed several times with the same medium and incubated in groups of 10 in small Petri dishes (35 mm in diameter) at 18 °C. If the incubation is to be extended overnight, Gentamycin (50 μg μl^{-1}) is added to the medium.

2.2.4. Extraction of GVs

Each oocyte is rinsed twice in Ca^{2+}-free "Isolation" medium (see below) and transferred to an embryo dish filled with the same solution to ensure that no Ca^{2+} is carried over [15]. Oocytes are dissected under higher magnification (1× to 1.25×) using two pairs of forceps (Dumont, No.5) by producing a tear within the animal hemisphere toward the vegetal hemisphere. Gentle pipetting is applied to the exposed surface of the cytoplasm using a glass pipette with an inner diameter of 0.5 mm as to make the GV appear and detached from the cytoplasm (Fig. 4). Yolk granules still adhering to the GV envelope can be removed by gentle back and forth movements of the GV in the pipette. During all these steps, the glass pipette must to be filled with "Isolation" medium so as to avoid any contact of the GV with air and make the back and forth movements better controlled. At this step, we prefer to use an embryo dish rather than a Petri dish because its thick bottom reflects the light allowing the GV to be better localized.

2.2.5. Dispersal of the GV content

As explained by Gall et al. [15] the purpose of this step is to transfer the nucleus from the isolation medium in which it would remain indefinitely as a gel, to a medium in which the gel will slowly disperse. Using a glass pipette filled with "Isolation" medium the GV is quickly transferred into the well of the "Dispersal chamber" previously filled with ca. 50 μl of "Dispersal" medium (Fig. 4). Care should be taken to let the GV go down gently into the well as to minimize the volume of "Isolation" medium added to the chamber. $MgCl_2$ must be added to the "isolation" and "dispersal" media for GV spreads to be used for the visualization of extrachromosomal structures such as nucleoli or Cajal bodies (see Appendix). In this case, special care should be taken not to leave the GV more than 1 min in the "Isolation" medium before transferring it to the "Dipersal" medium because the dispersal of its content would be difficult to obtain otherwise [18]. Fig. 5 shows the different aspects of the GV in the "Dispersal chamber" according to the stage of the oocyte from which they were isolated. The nuclear envelope is removed with forceps. The nuclear content may either form a dense gelatinous ball (early stages oocytes) or have the appearance of a transparent gel (late stages oocytes). The "Dispersal chamber" is left undisturbed on a tray placed on ice until complete dispersal of the nuclear content. This process may take 10–45 min according to the density of the nuclear sap and can be monitored using an inverted microscope with a low magnification phase-contrast objective (20×) before covering the well of the "Dispersal chamber" with an 18-mm coverslip. Great

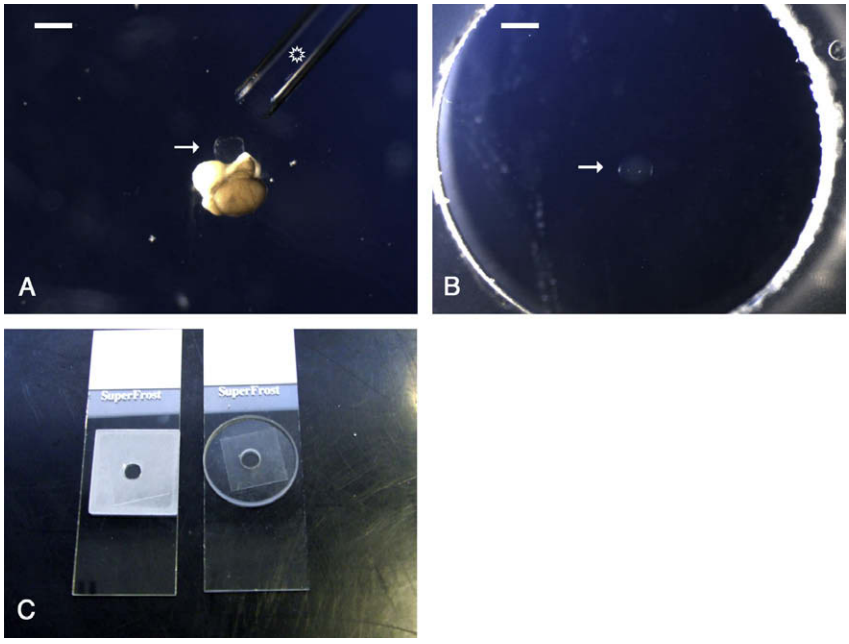

Fig. 4. Preparation of LBCs spreads. (A) Extraction of a GV (arrow). The glass pipette is indicated by a star. (B) a GV (arrow) in the "Dispersal chamber"; (C) two types of "Dispersal chamber"; (D) a top view of the centrifuge showing the arrangement of the microscope slides with their "Dispersal chamber" on cork plates. Scale bars represent 0.5 mm.

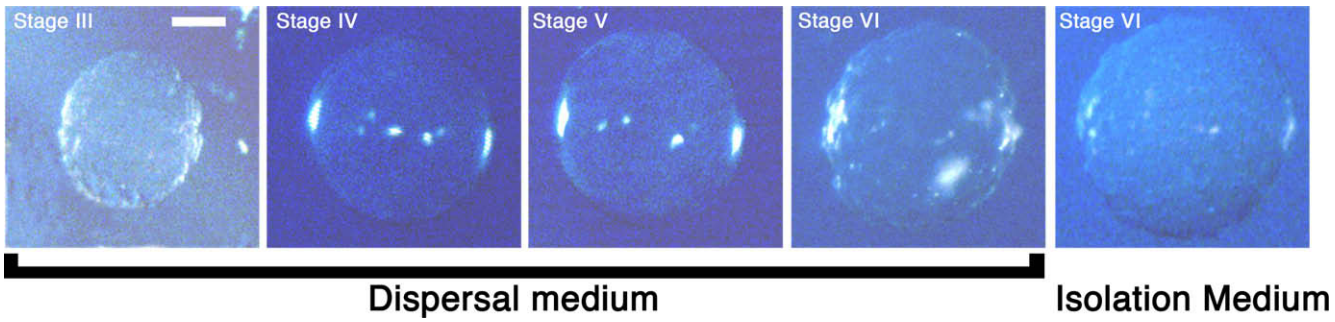

Fig. 5. GVs from stages III–VI oocytes in the "Dispersal" and "Isolation" media. Note that GVs from oocytes at different stages are transparent in the "Dispersal" medium, and opalescent in the "Isolation" medium as shown here for stage VI. Scale bar represents: 100 μm.

care should be taken to avoid the formation of air bubbles, which can disturb the GV spread when the coverslip is added. Firstly, the volume of "Dispersal" medium in the "Dispersal chamber" well should be adjusted (ca. 50 μl) as to make its surface slightly convex before transferring the GV. Secondly, the coverslip must be dropped onto the well and not slid onto it. Lastly, the excess of liquid should be absorbed by gently pressing a filter paper on the top of the "Dispersal chamber" without making the coverslip move.

As described in Table 1, this step differs slightly in our protocol from that of Gall et al. [15] for *X. laevis* LBCs in which the GV envelop is removed in a first Petri dish and the GV content "washed" in a second one filled with the "Dispersal" medium before transferring the GV gel to the "Dispersal chamber". Instead we have found that transferring the GV directly to the "Dispersal chamber", removing its envelope and waiting for the gradual dispersal of its content constitutes a safer way to

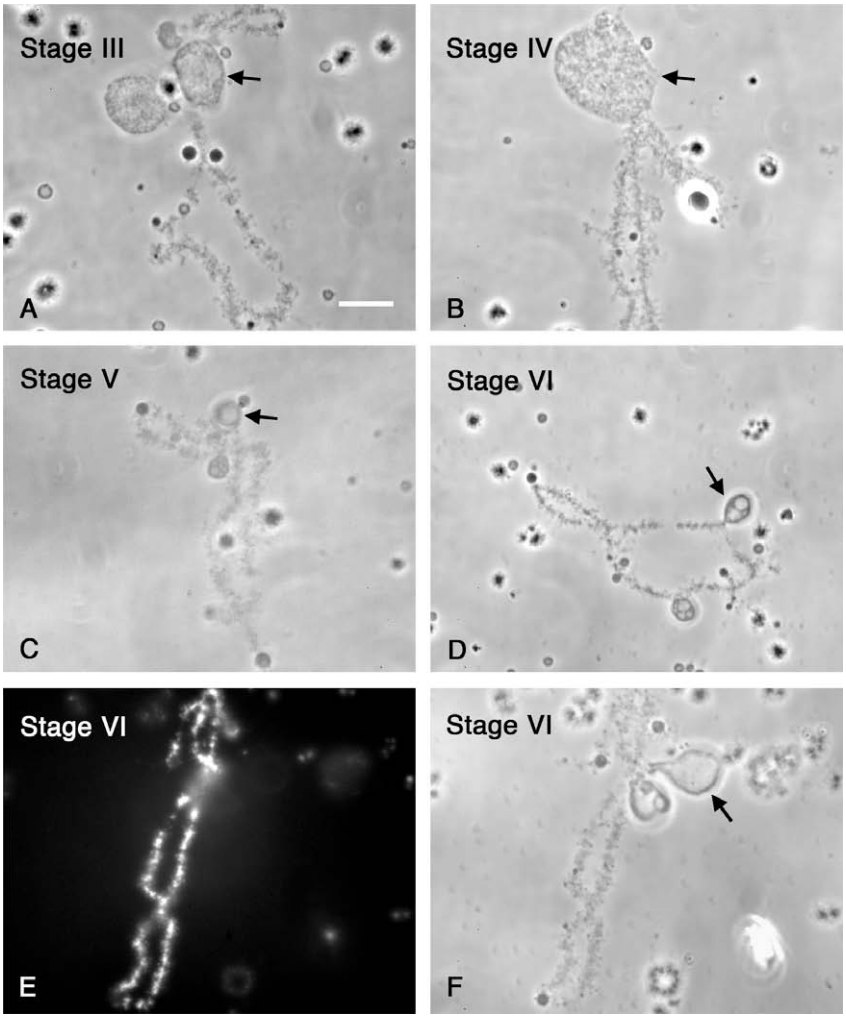

Fig. 6. Phase contrast (A–D, F) and fluorescent (E corresponding to F) micrographs of LBCs preparations from *X. tropicalis* fixed with paraformaldehyde, stored in 70% alcohol and stained with Hoechst (E) showing bivalent IV from stages III–VI oocytes. This bivalent can be identified by the presence of "Giant Fusing loops" at one of its extremities (arrows). (A–D) LBCs prepared from oocytes incubated *in vitro* for 24 h. Note that the bivalents are foreshortened and their lateral loops are less developed in stage V–VI oocytes than in stage III–IV oocytes. (E, F) Preparation from freshly isolated oocytes. Note that the bivalent in (D) is shorter than that in (F) indicating that *in vitro* incubation of the oocyte may cause a slowing down of the transcriptional activity. Scale bar represents: 10 μm.

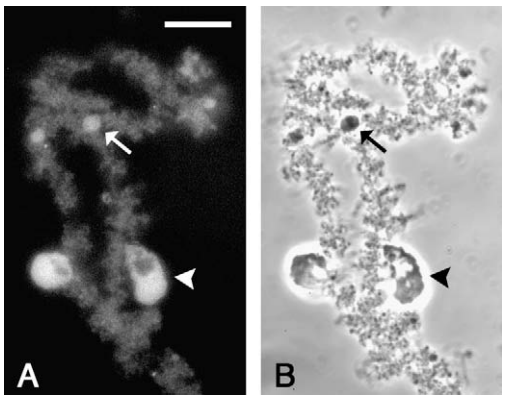

Fig. 7. Fluorescence (propidium iodine-staining) (A) and phase-contrast (B) images of LBCs spread preparation from an hormonally stimulated female showing a portion of bivalent II. This bivalent is identified by the presence of "Mega Fusing loops" (arrowheads) and lumpy structures (arrows). Note that the lateral loops are well developed and bulky. Scale bar represents: 10 μm.

prevent any loss of nuclear material caused by multiple transfers.

2.2.6. Centrifugation

This step is required to make the nuclear content firmly attached to the subbed glass slide before subsequent treatments. We use a refrigerated centrifuge with a swing-out rotor equipped with two sealed carriers for microplates (Fig. 4). The microscopes slides with their "Dispersal chamber" are arranged in groups of 4 on cork plates (8.5 × 13 cm) and fixed with adhesive tape. Each carrier may support at least two cork plates so that 16 preparations can be centrifuged at the same time. The slides are centrifuged at 300*g* for 10 min, and at 3100*g* for 30 min at 4 °C. After centrifugation, preparations are observed under phase contrast with an inverted microscope.

2.2.7. Fixation

LBCs preparations should be fixed when preserved for more than a few hours. Once fixed, GV spreads attached to the subbed

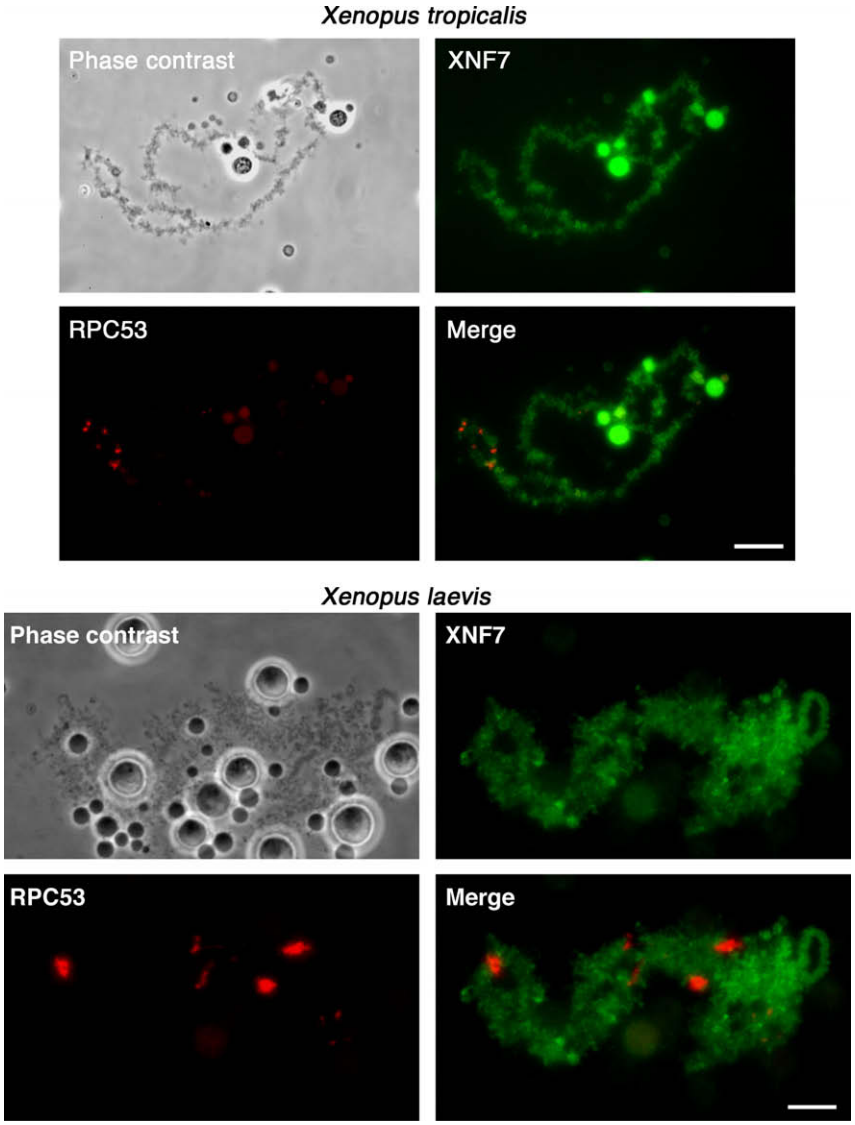

Fig. 8. Association of Nuclear factor 7 (NF7) with RNAPII transcriptional units. Phase contrast and corresponding fluorescent micrographs of LBCs preparations from *X. tropicalis* and *X. laevis* stained with anti-NF7 mAb 37-1A9 (green) and anti-RPC53 (red). mAb 37–1A9 stains loops that are also stained with antibodies against RNAPII [19], whereas anti-RPC53 is specific for RNAPIII [20]. Most chromosomal loops were labeled with mAb 37-1A9 (green), while only a small number of RNAPIII loci (red) are observed. Scale bars represent: 10 μm.

microscope slide are treated like other cytological preparations to be used for *in situ* hybridization or immunofluorescence assays. Fixation with 2% paraformaldehyde in phosphate-buffered saline (PBS) gives satisfactory results. A 70% alcohol solution can also be used as fixative or as post-fixative in which LBCs preparations may be stored for several days or weeks. Detailed fixation protocols were reported in Gall et al. [15], but we will describe here the main steps.

All operations are carried out while LBCs preparations are still immersed. After centrifugation, slides with LBCs preparations are placed horizontally in a large Petri dish filled with the fixative and the coverslip on top of the "Dispersal chamber" is carefully pushed off. Slides are fixed for 30 min at 4 °C and washed in PBS or in 70% alcohol. While the chamber is still immersed in the washing solution, the glass or plastic square that forms the "Dispersal chamber" is detached from the microscope slide, leaving the GV spread surrounded by a halo of paraffin wax. Alcohol-fixed preparations were dehydrated through an ethanol series, washed in xylene to remove paraffin wax and post-stained with Coomassie-blue R. Immunofluorescence assays are generally processed using preparations freshly fixed with paraformaldehyde or preparations fixed with paraformaldehyde and stored in 70% alcohol. In the latter case, rehydration through 50% and 35% alcohol to PBS should be carried out before use. Paraformaldehyde preparations are post-stained with either Hoechst 33342 (1:1000), 1 μg/ml of 4,6-diamidino-2-phenylindole (DAPI) or propidium iodide (10 μg/ml).

3. Specific recommendations

The purpose of this protocol is to maximize the yield of LBCs with easily identifiable chromosomes displaying well-developed lateral loops. LBCs are shorter and their lateral loops are less developed in stages V–VI oocytes than in stages III–IV oocytes reflecting a slowing down of transcription. This decrease in transcription may also be observed after 24 h of *in vitro* incubation, especially with stage V–VI oocytes (Fig. 6). However, the fact that oocytes withstand *in vitro* incubation for a long period of time allows the preparation of LBCs to be extended over 2 days from a single ovary fragment. With some practice 30 LBCs preparations can be routinely obtained per day. The possibility to store LBCs preparations in 70% alcohol without altering their structure allows them to be used several days or weeks later.

In the ovary of hormonally stimulated females, the number of stage VI oocytes is usually lower than that in non-stimulated females. Hormonal stimulation enhances transcriptional activity, allowing LBCs with well-developed lateral loops to be isolated from all oocyte stages including stage VI (Fig. 7). However, hormonal stimulation is dispensable because ovaries of non-stimulated females contain a sufficient number of stages III–V oocytes for carrying out a large number of LBCs spreads with one biopsy.

4. Advantages and limitations of the use of LBCs preparations of *X. tropicalis*

It has been admitted for a long time that the relative length of lateral loops in one species can be directly correlated to the *C* value of its genome (for review, see [6]). The lampbrush loops of *X. laevis* are shorter than those of *Rana* (*C* value 10 pg), which are shorter than those of some salamanders such as *Pleurodeles waltl*, *Notophthalmus viridescens* or *Triturus cristatus* with *C* values ranging from 30 to 50 pg (cited in [15]). Similarly, lampbrush loops of *X. tropicalis* are less developed and less bulky than those of *X. laevis* with a larger genome size (3.1×10^9 vs. 1.7×10^9 bp) and their LBCs are smaller (chromosome axial length ranging from 17 to 47 μm in *X. tropicalis* [8] vs. 75 to 160 μm in *X. laevis* [12]).

Despite their small size, *X. tropicalis* LBCs can be used like those of *X. laevis* LBCs for cytological analyses of transcriptional processes (Fig. 8). The main interest of using *X. tropicalis* LBCs will be to contribute to the understanding of the organization of the amphibian genome and the mapping of a variety of loci of interest. Compared to *X. laevis*, which is allotetraploid, *X. tropicalis* is diploid with 10 chromosomes instead of 18. This small number of LBCs and their small size constitute a clear advantage for the preparation of GV spreads in which the chromosomes are well separated from one another facilitating their identification.

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Appendix A

A.1. Working solutions

The detailed preparation of working solutions and the effect of their different ingredients on the structure of amphibian LBCs were described in detail by Gall et al. [15]. We give here complementary information for the preparation of LBCs spreads from *X. tropicalis* oocytes.

- *OR2 buffer (Oocyte Ringer's medium)* [17]:82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mMNa_2HPO_4, 5.0 mM HEPES, pH 7.5.
- *MBS buffer (Modified Barth's solution)* [16]*:* 80 mM NaCl, 1 mM KCl, 0.7 mM $CaCl_2$, 0.8 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 1 mM HEPES, pH 7.5.
- *"Isolation" medium used for LBCs of X. tropicalis:* "5:1 + PO_4^{3-}":83 mM KCl, 17 mM NaCl, 10 mM PO_4^{3-}, pH 7.2":a Ca^{2+}-free solution to keep the nuclear content as a gel indefinitely. PO_4^{3-} has a specific gelling effect.
- The "Isolation" medium used for LBCs of *X. laevis* is the same medium as above supplemented with 1 mM $MgCl_2$.
- *"Dispersal" medium used for LBCs of X. tropicalis:* 25% of "5:1 + PO_4^{3-}"+ 10 μM Ca^{2+} + 0.1% w/v paraformaldehyde. The micromolar concentration of Ca^{2+} destabilizes the nuclear content and leads to its dispersion. The low paraformaldehyde concentration also helps to destabilize the nuclear gel but stabilizes the loops.
- The "Dispersal" medium used for LBCs of *X. laevis* is the same medium as above supplemented with 1 mM $MgCl_2$ + 1 mM dithiothreitol (DTT). The addition of Mg^{2+} is required for the stabilization of extrachromosomal elements (nucleoli, Cajal bodies).
- *Subbing solution:* 0.5% (w/v) Gelatin (Type A, G2625, Sigma), 0.05% (w/v) Chrome alum ($CrK(SO_4)_2{\cdot}12H_2O$). Gelatin is dissolved in hot water, cooled down before adding the Chrome alum while stirring.

A.2. Experimental materials

- *Gelatin-subbed slides:* clean, dry slides are briefly dipped in fresh subbing solution, drained separately in an appropriate carrier and air-dried over night or until complete drying. When handling the subbed slides care should be taken not to scratch the gelatin surface. Subbed slides should be stored at 4 °C and can be used during 30 days.
- *Dispersal chamber:* this chamber consists of a subbed microscope slide onto which a 25-mm disk of 1 mm-thick glass or a square of Plexiglas with a 5 mm hole bored in the center is sealed with 45 °C m.p. paraffin wax (Fig. 4). Such a superposition forms a small well into which the dispersal of the nuclear content takes place.
- *Glass pipettes:* Pasteur pipettes stretched to a diameter of 0.5–0.8 mm are used.

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