

Chapter 4

Working with Oocyte Nuclei: Cytological Preparations of Active Chromatin and Nuclear Bodies from Amphibian Germinal Vesicles

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Abstract The giant nucleus or germinal vesicle (GV) of amphibian oocytes presents a remarkable opportunity to examine nuclear structures in unprecedented levels of detail. By making use of spread preparations of GVs, it is possible to investigate the structure and function of transcription units in active chromatin and a variety of nuclear bodies, all within the limits of resolution of the light microscope. The basic method for producing GV spreads that is described here is based on simple manual dissection and, therefore, it permits the preparation of nuclear components that have suffered a minimum of experimental manipulation. The particular method described is based on the use of oocytes from a salamander, the axolotl, although the approach is robust and applicable with minor modification to two other model amphibian species, *Xenopus laevis* and *X. tropicalis*. One common approach to investigating the molecular organisation of oocyte nuclear structures by immunofluorescent staining of endogenous or exogenous polypeptides is also described.

1 Introduction

A combination of factors, such as its large size (up to 1 mm diameter), a massive rate of gene activity, and the ease with which its contents can be prepared in a spread-out form has meant that the giant nucleus or germinal vesicle (GV) of the amphibian oocyte can provide exceptionally favourable material for cytological studies of nuclear structure and function, particularly in relation to the control of gene expression. The basic procedure for making spread preparations for light microscopy that is described in this chapter has remained fundamentally the same for many years, and such preparations continue to be the most commonly used approach for studying GV contents. Historically, however, conventional sectioning of oocytes and the preparation of GV contents for electron microscopy in the manner described by Ann Beyer's group (Vol. 2; Chap. 4) have also contributed important

discoveries (reviewed in **ref. (1)**), while, in the future, nondisrupted GV whole mounts made in oil will undoubtedly be valuable for live cell approaches (**2, 3**).

The key attribute of GV spread preparations is the presence of a range of nuclear structures with extraordinary levels of morphological detail. The best known of these are the giant lampbrush chromosomes (reviewed in **ref. (4)**), which possess extended chromatin loops that are transcribed by RNA polymerase II (pol II) at far greater rates than are most genes in somatic nuclei. Individual transcription units can extend up to several hundred microns in length and their transcriptional polarity can be deduced by phase contrast microscopy simply from the asymmetrical distribution of the large mass of nascent ribonucleoprotein (RNP) associated with the transcribed DNA. This remarkable form of chromatin therefore permits the analysis of transcription sites with a clearly defined structure and provides in situ access to the processes operating during the elongation stage of transcription, all within the limits of spatial resolution of the light microscope. For example, recent studies of lampbrush transcription units have used immunofluorescence microscopy to detect either endogenous or epitope-tagged, exogenous polypeptides in injected oocytes to investigate the composition of nascent RNPs and the disposition of pol II phosphoisomers in highly active chromatin (reviewed in **ref. (4)**). Active chromatin of another type is found in amphibian GVs in the form of numerous extrachromosomal amplified nucleoli, and these structures have long been useful for the study not only of pol I transcription but also of the processing of ribosomal RNAs and other aspects of nucleolar function (see, for example, **refs. (5, 6)**). Other structural components of the amphibian GV have recently been studied in detail by J.G. Gall and collaborators (reviewed in **ref. (7)**). Most notable are B-snurposomes, which are thought to be homologous to splicing speckles/interchromatin granule clusters, and a second type of GV body that is the counterpart of the somatic cell Cajal body (CB). Both of these nuclear bodies are especially amenable to study in GVs because they are larger and more numerous than the somatic equivalents (reviewed in **refs. (8, 9)**).

Preparation of GV spreads comprises just three basic steps: first, a GV is isolated by hand from an oocyte into an isotonic saline. Second, the nuclear envelope is removed, also manually, in a lower ionic strength saline and the gelatinous GV "sap" encouraged to disperse in a small chamber formed on a microscope slide. Finally, the larger macromolecular components of the dispersed GV contents are attached to the slide by centrifugation in preparation for subsequent manipulations such as fixation and immunostaining. Significantly, soluble nucleoplasmic components are rinsed off during the latter manipulations. Detailed and subtle variations on this basic technique have been introduced over the years as oocytes from different amphibian (and nonamphibian, e.g., **ref. (10)**) species have been studied. The physical and chemical principles underlying many of the elements of the basic technique and its variants have been described authoritatively several times (**1, 11, 12**) and will not be repeated in detail here. Many of the variations were introduced to allow the dispersal of GV saps that are notably more viscous than others, a property that can be species specific or oocyte stage dependent. Low concentrations of formaldehyde and of calcium in the dispersal media are key ingredients that permit the dispersal of the

more viscous GVs. Recently it has become clear that a major determinant of GV sap viscosity, namely the unusually high concentration of nuclear actin, results from the absence of cognate nuclear export receptors ((13); reviewed in ref. (14)).

Although GV spread preparations have been made successfully from many animals, the best material for studies of lampbrush chromosomes is provided by the oocytes of tailed amphibians (urodeles), particularly the newts *Triturus spp*, *Notophthalmus viridescens*, and *Pleurodeles waltl*. These species have dominated research using GV spreads for two main reasons: first, compared with the other main group of amphibians, the frogs and toads (anurans), urodele oocytes possess lampbrush chromosomes in which the transcriptionally active loops are more highly extended. Moreover, unlike anurans and even other urodeles, GVs from those stages of oocyte development in which the chromosomes have well-developed loops are also relatively easy to disperse in newts, contributing to technically superb preparations. However, recently, two problems with the use of newt material have become significant impediments and have led to the increasing use of other amphibian species. First, newts are increasingly difficult to collect from the wild; indeed, many species are now protected and they are not easily bred in the laboratory. Secondly, related molecular reagents and bioinformatics resources are extremely limited.

Neither of these shortcomings applies to the amphibian model organism that has been studied in countless investigations in molecular, cell and developmental biology, namely, the South African clawed frog, *Xenopus laevis*. Although the more viscous *Xenopus* GV sap has long presented a challenge to the preparation of spreads, recent efforts by J.G. Gall and colleagues have led to the refinement of the spreading procedure to overcome the difficulties in its dispersal (15). However, it still remains the case that the underdevelopment of the transcriptionally active loops in *Xenopus* lampbrush chromosomes makes them less than ideal for studying active chromatin in situ, although the nuclear bodies of the *Xenopus* GV are currently the best characterised both in spreads and in “live” nuclei (2, 3). Fortunately, as described here in detail, the improved approach detailed by Gall (15) for dispersing the stiff GV sap of *Xenopus* can also be applied essentially unchanged to the preparation of spreads from a species of salamander that has similarly viscous GV sap but that, as a urodele, possesses superbly developed lampbrush chromosomes. Moreover, because of its emergence as the model urodele species for embryological and regeneration studies, this salamander, *Ambystoma mexicanum*—also known as the axolotl—does not exhibit the problems of supply and resources noted above for newts.

Although not often used for studying GVs, *Ambystoma* has made occasional but distinguished appearances in the history of such research; lampbrush chromosomes were first described in sections of *A. mexicanum* oocytes by Flemming in 1882 (16), and *A. tigrinum* oocytes were among those used by Gall (17) in his reinvention of lampbrush studies in 1954. Fortunately, axolotl lampbrush chromosomes and nucleoli were also the subject of a later, definitive study by Callan (18), and the GVs of *A. macrodactylum* have received similar attention from Kezer and colleagues (19). Hence, there is a small but authoritative body of work on *Ambystoma* GV structures that, particularly with regard to the identification of individual lampbrush chromosomes, is very helpful. The re-introduction of axolotls for preparing

GV spreads is however mainly driven by this species' emergence as a model organism; in effect, it is the urodele equivalent of *Xenopus* with regard to 1) the availability of stocks and ease of establishment of laboratory colonies (20), and 2) the development of extensive EST sequencing projects and bioinformatics resources (21, 22). A final point is that, as noted below, essentially the same approach described here can be applied to prepare active chromatin and nuclear bodies from oocytes of *Xenopus (Silurana) tropicalis*, a species that is becoming of increasing interest because of the potential application of genetic approaches and, uniquely among amphibians, an ongoing genome sequencing project (23).

2 Materials

2.1 GV Isolation and Dispersal

1. OR2 medium: 82.5 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgCl₂, 1.0 mM Na₂HPO₄, and 5.0 mM HEPES (from a 0.5 M stock, pH 8.3). Final pH 7.4–7.8. Store at 4°C.
2. GV isolation medium: 83 mM KCl, 17 mM NaCl, 6.5 mM Na₂HPO₄, 3.5 mM KH₂PO₄, and 1 mM MgCl₂, stored at 4°C. Just before use, add dithiothreitol (DTT) to 1 mM from a 1.0 M frozen stock, and filter through a 0.45-µm nitrocellulose filter. Final pH 7.0–7.2.
3. GV dispersal medium: GV isolation medium stock diluted to 25% (final concentrations 20.8 mM KCl, 4.3 mM NaCl, 1.6 mM Na₂HPO₄, and 0.9 mM KH₂PO₄) with MgCl₂ adjusted to 1.0 mM, 0.01 mM CaCl₂, 0.1% (w/v) paraformaldehyde (from a 20% stock; see Sect. 2.2.3; handle in a fume hood). Final pH 7.0–7.2, adjusted with 100 mM Na₂HPO₄ if necessary. Stored at 4°C. Just before use add DTT to 1 mM from a 1.0 M frozen stock, and filter through a 0.45-µm nitrocellulose filter.
4. Dispersal chambers: chambers based on standard microscope slides consist of a gelatin-treated (subbed) slide onto which is fixed a square of Perspex (Plexiglas; 24×24×1.5-mm thick) that has a 6-mm-diameter hole drilled in its centre. The plastic square is stuck temporarily to the centre of the slide using a few drops of a 1:1 mixture of Vaseline (petroleum jelly) and paraffin wax (solidification point 51–53°C). To sub microscope slides, immerse clean, dry slides in a freshly prepared and filtered (#1 paper, Whatman, Maidstone, UK) solution of 0.1% (w/v) gelatin, 0.01% (w/v) CrK(SO₄)₂ and drain them before drying overnight at 65°C. If equipment allowing the centrifugation of microscope slides is not available, chambers based on round coverslips can be used (see Note 1).
5. Instruments for GV manipulation and dissection: three or four pairs of Dumont #5 watchmakers' forceps, preferably not stainless steel, so that their points can be finely sharpened by hand. A sharp tungsten needle mounted in a pencil-sized piece of glass tubing provides a convenient tool for dissecting the GV envelope. Moving the GV between solutions requires Pasteur pipettes (150 mm) that have been stretched in a Bunsen flame from just above the shank, such that the

stretched section can be broken to produce a capillary about 6-cm long with a diameter of about 0.8–0.9 mm at the tip. The broken tip should be polished in a small flame to remove sharp edges that would damage the GV envelope. Use 2-mL rubber teats to aspirate solutions and GVs.

6. Coverslips: 18×18 mm, no. 1½.
7. Petri dishes: 100-mm and 35-mm diameter.

2.2 Attachment and Fixation of GV Contents

1. Carriers for centrifugation of dispersal chambers. These can be either custom-made slide holders as described by Gall et al. (12) that are designed to fit the swing-out rotor of a high-speed centrifuge (e.g., the HS-4 rotor for a Sorvall RC-5C), or any swing-out centrifuge bucket or plate holder that is large enough to accommodate a microscope slide and is rated for an RCF of 5,000. If either option is unavailable, disc-shaped dispersal chambers can be used that are accommodated by a variety of centrifuge tube and rotor combinations (see Note 1).
2. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.5 mM KH₂PO₄; prepared and autoclaved as a 20× stock solution. After dilution, add MgCl₂ to 1 mM.
3. Paraformaldehyde: 20% (w/v) paraformaldehyde (Sigma-Aldrich, Gillingham, UK) stock solution made up in 4 mM Na₂CO₃. In a fume hood, carefully heat on a stirring hot plate to 60°C to dissolve and after cooling filter through Whatman #1 filter paper. Store at 4°C. Prepare fixative by diluting to 2% (v/v) in PBS.

2.3 Immunostaining Fixed GV Spreads

1. Immunoblocking and antibody dilution buffer: 5% (w/v) normal goat serum (NGS) (Jackson Immunoresearch Laboratories, Newmarket, UK) in PBS.
2. DNA stain: 4,6-diamidino-2-phenylindole (DAPI), 1 mg/mL stock stored at 4°C and diluted to 0.5 µg/mL in PBS.
3. Mounting medium: 50% (v/v) glycerol diluted with PBS.

3 Methods

The technique described below is one which I have used successfully to prepare GV spreads from axolotl oocytes. It is directly derived from, and differs in only minor ways from, the latest technique developed by Gall for *Xenopus laevis* oocytes (15); the variations applicable to the latter are described in the Notes section. Indeed, this robust technique can probably be successfully applied to the preparation of GV spreads from almost any amphibian oocyte, particularly those with a more gelatinous GV sap. For instance, the procedure described is equally applicable to making GV spreads from *Xenopus tropicalis*.

3.1 *GV Isolation and Dispersal*

1. Ovaries freshly removed from an anaesthetised axolotl are placed in OR2 medium at 18–22°C in a 10-cm diameter Petri dish and, using watchmakers' forceps, are divided into fragments consisting of clumps of 50 to 100 oocytes. Once divided, any damaged oocytes are removed and the ovary fragments transferred to fresh OR2, with only five to six clumps per dish. Oocytes can be used immediately for GV spread preparations, or they can be stored at 18–22°C for several days, provided unhealthy oocytes or their contents are regularly removed from the medium (*see Note 2*).
2. Solutions required for GV isolation, washing, and dispersal are allowed to come to room temperature and are made ready on or close to the stage of a dissecting microscope. Isolation and washing are performed in prefilled 35-mm plastic Petri dishes that are clean but have been "seasoned" by multiple uses in this procedure. A dispersal chamber is filled with about 50 μ L of medium such that a slight convex meniscus forms. The stretched Pasteur pipettes for handling GVs are charged with isolation or dispersal media that should be completely free of any air bubbles.
3. Transfer to a dish of isolation medium a small clump of oocytes containing some in the optimal size range for lampbrush chromosome preparation (usually 1.3–1.7 mm diameter, i.e., equivalent to stage V (24); *see Note 3*). Using two pairs of sharp watchmakers' forceps, a suitable oocyte is torn apart from the vegetal toward the animal pole. The spherical GV will often be seen as a partial clearing in the continuous film of yolk and should immediately be sucked into a pipette containing isolation medium. The GV should then be transferred swiftly (within 10–20 sec), and with as little yolk as possible in the time frame, into a dish containing the dispersal medium. In all GV transfers, it is crucial to avoid the presence of air bubbles and sharp or broken edges on the pipette, to prevent premature rupture of the GV.
4. Using a second pipette filled this time with dispersal medium, the GV should be transferred in a small volume and with minimal yolk platelets to a prefilled dispersal chamber. In addition to allowing yolk removal, the wash step enables transfer of the GV into the dispersal chamber without an increase in the low salt concentration of the dispersal medium. Again, washing and transfer steps should be completed quickly because this obviates swelling of the GV, which makes subsequent handling difficult. Immediately after transferring the GV to the chamber, pick it up with a pair of watchmakers' forceps to create a firm grip but not puncture the nuclear envelope (lateral illumination from a cold light source and a black background provide the best conditions for observing the GV in the chamber). Holding the GV just above the surface of the chamber, the envelope should be torn open over a third to a half of its circumference using either the finely sharpened point of a tungsten needle or a second pair of forceps. The aim is just to tear the envelope, but not to penetrate beyond the envelope and disrupt the underlying GV contents. The forceps holding the punctured GV should be held perfectly still for as

long as it takes the GV contents to spill out of the envelope; depending on the oocyte, this may take from a few seconds to about a minute. When the last of the GV sap has been released (which may occur quite suddenly as if a physical connection has given way), withdraw the remnants of the envelope, leaving the GV contents as an undisturbed gelatinous mass that will become more liquid and flatter as dispersal continues. As the forceps are taken through the surface of the medium, the remnants of the envelope that they hold will disintegrate.

5. Place a coverslip over the observation chamber and seal the edges with molten Vaseline (petroleum jelly). Observe the extent of dispersal and flattening of GV contents under an inverted microscope fitted with a low-power phase contrast objective (e.g., $\times 16$). It may take between 20 min and 1 h for the lampbrush chromosomes and GV bodies all to lie in the same plane on the surface of the slide. See Fig. 4.1 for the typical appearance of an axolotl lampbrush chromosome at this stage of preparation, i.e., after dispersal but prior to centrifugation and fixation.

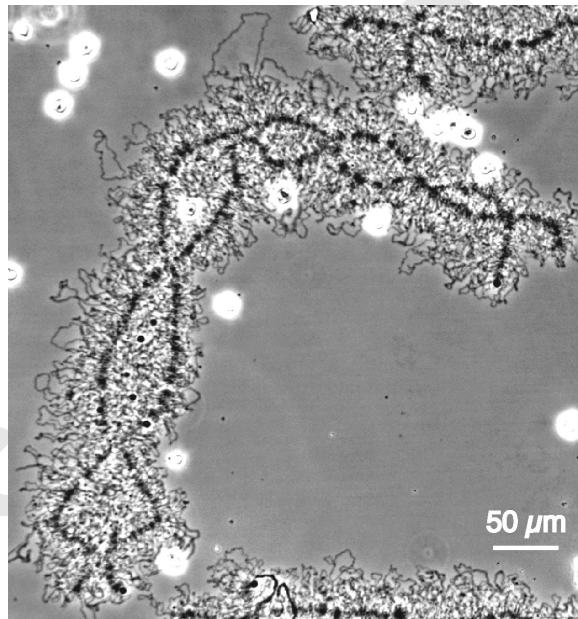


Fig. 4.1 A medium-sized lampbrush chromosome freshly isolated from an axolotl oocyte and observed at low magnification by phase contrast microscopy. The nuclear gel has fully dispersed and the chromosome is lying on the surface of a dispersal chamber awaiting the subsequent centrifugation and fixation steps necessary to permanently attach and preserve the spread chromatin. Prior to their attachment to the chamber surface, the numerous transcriptionally active lateral loops projecting from the axes of this meiotic bivalent are in constant Brownian motion. The rather globular, refractile objects are extrachromosomal, amplified nucleoli

3.2 Attachment and Fixation of GV Contents

1. Once lying flat on the bottom of the slide chamber, the GV structures must be centrifuged to attach them firmly. Transfer the dispersal chambers to an appropriate carrier for centrifugation (*see Note 1*) and, using a slow-start setting, centrifuge at $5,000\times g$ for 45 min at 4°C . After centrifugation, preparations can be monitored under an inverted microscope prior to fixation.
2. In a fume hood, place the slides vertically into a staining dish containing 2% paraformaldehyde in PBS and, using forceps, gently push to one side the coverslip covering the observation chamber. Leave the preparations to fix for 1–2 h (or less where appropriate for certain antibodies) and then prise off the bored Perspex square from the slide with a razor blade.
3. Remove the slides from the staining dish and rinse in PBS. Preparations can then be immunostained immediately or stored in PBS at 4°C for several weeks for later immunostaining if necessary; alternatively, they could be processed for other purposes such as in situ hybridisation at this stage (**Note 6**).

3.3 Immunostaining Fixed GV Spreads

Because GV spreads comprise simply the chromatin and nuclear bodies from only a single nucleus, the absence of nucleoplasmic and cytoplasmic components contributes to very low background levels even when small volumes of reagents are used for immunostaining. Moreover, the presence of a dam of paraffin wax surrounding each spread also allows for economy of solutions used; it is feasible to use volumes of primary antibody of less than $10\mu\text{L}$. Incubation of slides should then be carried out in a closed chamber moistened with PBS to prevent the preparation drying out.

1. Incubate the preparation in $100\mu\text{L}$ of 5% NGS for 15 min and then add primary antibody diluted in the same for 1 h.
2. Wash $3\times$ in $100\mu\text{L}$ of 5% NGS and add secondary antibody diluted in PBS for 1 h.
3. Wash $3\times$ in PBS with the penultimate wash containing DAPI at $0.5\mu\text{g}/\text{mL}$ if desired. The intense DAPI staining of lampbrush chromosome axes makes it simpler to identify and follow individual bivalents than in preparations viewed only by phase contrast or differential interference contrast (DIC) microscopy (Fig. 4.2).
4. Cover the preparation in $20\mu\text{L}$ of 50% glycerol/PBS, use a razor blade to scrape away any paraffin wax from around the spread, and mount with an 18-mm square coverslip. An immunostained GV spread from an axolotl oocyte is shown in Fig. 4.3.

Fig. 4.3 (continued) fluorescent images are merged, one comprising the preparation immunostained for RNA polymerase II (pol II) (*green*), and the other the results of DAPI staining (*blue*). Immunostaining utilised the monoclonal antibody H14, which detects a specific RNA polymerase phosphoisomer; staining is apparent throughout the length of the lateral loops and is particularly intense in Cajal bodies and suspended granules, although in the other nuclear bodies it is undetectable

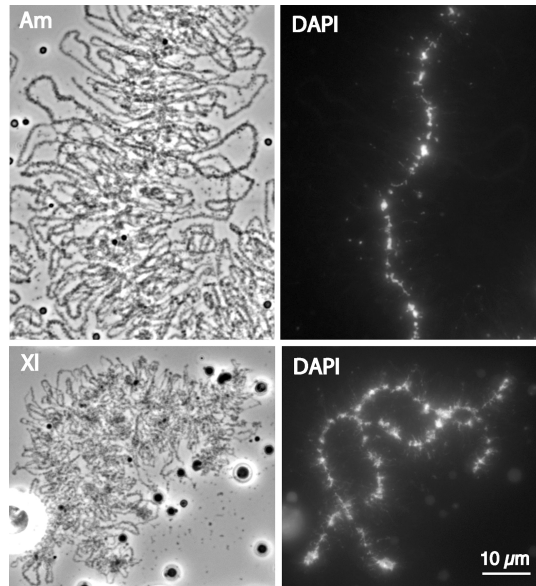


Fig. 4.2 Lampbrush chromosome spread preparations after fixation and DAPI staining viewed at high magnification by phase contrast (*left panels*) and fluorescence (*right panels*) microscopy. In the *top panels*, a small portion of a lampbrush chromosome from an axolotl (*Ambystoma mexicanum* (*Am*)) is shown to the same scale as an entire lampbrush bivalent from a frog (*Xenopus laevis* (*XI*)) in the bottom panels. As in these examples, when made from oocytes in which the loops are highly extended, the large mass of loop material makes it difficult to discern the chromosome axes without the aid of DAPI staining. As well as the lampbrush chromosomes, GV bodies such as a nucleolus and many B-snurposomes (or interchromatin granule clusters) are visible in the *Xenopus* image

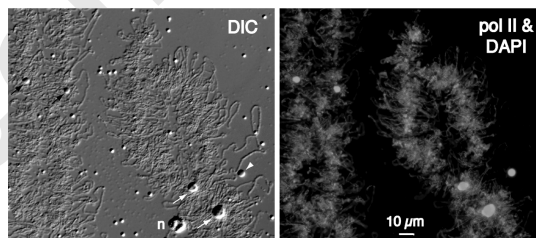


Fig. 4.3 Fixed and immunostained GV spread from an axolotl oocyte. The *left panel* shows portions of two lampbrush bivalents observed by differential interference contrast (DIC) microscopy, which is better suited than phase contrast microscopy for observation of nuclear bodies. As well as the actively transcribed chromosomal loops, two types of body that are regularly attached to axolotl lampbrush chromosomes can also be seen. The *white arrows* indicate Cajal bodies at the two loci on chromosome 13 described by Callan (18), and the *black arrows* indicate structures that he termed “suspended granules” that are found at homologous positions on many of the chromosomes. Free nuclear bodies are also visible including a Cajal body (*white arrowhead*), a nucleolus (*n*), and many smaller bodies equivalent to the B-snurposomes shown in Fig. 4.2. In the *right panel* two

[Au1] 4 Notes

1. Spread preparations can be made in disc-shaped dispersal chambers that do not require access to the more specialised centrifugation carriers needed for slide-based chambers. Discs 24-mm in diameter are made from 1.5-mm thick Perspex and a 6-mm hole is drilled in the centre. Again use wax to fix a 22-mm diameter, no. 1 1/2 coverslip to the plastic disc and, because the coverslip will form the base of the chamber, prior subbing of its surface (as described above for slides) will enhance the retention of GV material. Once a GV has begun dispersal in the chamber, it is covered with a 19-mm round coverslip for observation and centrifugation. These disc-shaped chambers should be compatible with many standard tube and carrier combinations for swing-out rotors (e.g., 50-mL polycarbonate tubes for the 00480 carriers of the Sorvall HS-4 rotor). Make a flat bed for the disc chamber by putting an appropriately sized rubber stopper into the bottom of the tube or by casting a similarly sized plug of epoxy resin; a hole drilled in the bottom of the tube will allow the insertion of a metal rod in order to lift or lower the stopper or resin plug and thereby facilitate loading and unloading the disc chamber. Although undoubtedly more demanding to handle than slide-based preparations, especially when prising apart the chamber, coverslip-based GV spreads offer the advantage to those experienced in working with cells cultured on coverslips of being amenable to the same containers and techniques for immunostaining, etc.
2. When using *Xenopus* oocytes, allow an overnight period (18–24 h) of recovery after ovary removal before attempting to make GV spreads (15). This period allows for the maximal extension of lampbrush loops. Incidentally, when preparing defolliculated oocytes for micro-injection, it is often useful to incubate them overnight prior to injection in order to identify any oocytes damaged by the defolliculation procedure. Obviously this incubation period, as well as any involved in an injection protocol, will also constitute the recovery period for GV spread preparations that are made from injected oocytes.
3. In particular when studying lampbrush chromosomes, selection of the optimal stage of oocyte development from which to prepare GV spreads is a compromise. On the one hand, the levels of synthetic activity and hence the extent of chromatin decondensation and loop extension are more marked in earlier stages, but on the other hand the speed and completeness of GV dispersal and the production of untangled, unbroken chromosomes are difficult in early stages. The optimal stage and size of oocyte is also a characteristic of the particular amphibian material to be used. For *Xenopus laevis*, pick oocytes in late stage IV (25) to early stage V (i.e., ~0.8 to 1.1 mm in diameter), while for the smaller frog *X. tropicalis*, I have found that oocytes of 0.5–0.6 mm are optimal for GV spreads.
4. The rapidity of the response of *X. laevis* GVs to gelling in aqueous solutions, coupled with the shorter lampbrush chromosomes, means that a slight modification of the procedure described above is recommended (15). The difference is that the GV envelope is removed in the dish of dispersal solution used for washing and *then* the isolated sap is transferred to the dispersal chamber. This aids rapid dissolution of the GV sap and spreading of the nuclear contents. The utmost speed in nuclear isolation, GV envelope removal, and transfer to the dispersal chamber are still of the essence in achieving satisfactory spreading of the GV contents, although the resultant dispersal periods can be much shorter than those for axolotl GVs (just a few minutes). However, in axolotls the characteristics of the GV sap, particularly its initial apparent physical connection to the envelope, and the presence of longer and more delicate chromosomes occupying more of the GV volume, both mean that this adaptation is not helpful for axolotl GV spreads (nor for *X. tropicalis*, in my experience).
5. In some individual axolotls, the GV sap may be less viscous than in others, and the concentration of formaldehyde in the dispersal solutions can be reduced to 0.01%, which may help in ensuring firm attachment of the GV material to the dispersal chamber.
6. Residual paraffin wax will normally mark the position of the well in the observation chamber and surround the attached GV contents. This dam of wax allows the preparation to be

temporarily mounted in PBS and the coverslip later removed without damaging the spread. It is useful when initially developing the procedure to check preparations at this stage by phase contrast or DIC microscopy in order to assess the efficacy of spreading, attachment, and preservation prior to immunostaining. The coverslip is then simply floated off in PBS before subjecting the preparation to further processing.

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[Au2] References

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Author Queries:

[Au1]: Notes 4 and 5 do not seem to be cited in the text. Please correct if necessary.

[Au2]: References 20–25 renumbered to match text citation order. Pleaes check carefully.