Isolation and analysis of *Xenopus* germinal vesicles.

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Abstract

The giant nucleus or germinal vesicle (GV) of *Xenopus* oocytes provides an unusual opportunity to analyse nuclear structure and function in exquisite detail by light microscopy. Detailed here are two rapid procedures for using manually-isolated GVs in combination with fluorescent reporter proteins to investigate the lampbrush chromosomes and nuclear bodies of oocytes. One procedure provides spreads of nuclear components in an unfixed and life-like, though not living, form. The other describes the isolation of intact, functional GVs directly into mineral oil offering possibilities for direct observation of nuclear dynamics.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

GV dispersal medium <R>

GV isolation medium <R>

Mineral oil (Sigma M5904)

Modified Barth's/GTP Saline (MBS) <R>

Paraffin wax (solidification point 51-53°C)

Vaseline (petroleum jelly)

Xenopus laevis ovary

Equipment

Dispersal/observation chambers

Use either a microscope slide or Perspex disc (24 mm diameter, 1.5 mm thick Plexiglas) with a 6 mm diameter hole drilled in the centre. To form the floor of the chamber seal a coverslip over the hole using a molten drop of a 1:1 mixture of Vaseline and paraffin wax. See diagram (Fig. 1D).

Filter paper (Whatman #1)

Glass coverslips (18x18 mm; No. $1^1/_2$)

Glass microscope slides (standard)

Inverted and upright fluorescence microscopes.

Inverted phase contrast microscope

Differential interference contrast (DIC) microscope (optional; see Step 8)

Pasteur pipettes (glass; 150 mm)

Pull some glass Pasteur pipettes in a Bunsen flame from just above the shank to produce a narrower capillary section that can be broken at about 6 cm from the shank so as to leave a tip with a diameter of 0.8-0.9 mm. Use a 2 ml rubber teat to aspirate GVs in and out of the capillary section of these pipettes.

Petri dishes (plastic; 35 mm diameter).

Stereo microscope and fibre optic light source

Syringe needle (25G)

Tungsten needle (sharpened)

Watchmakers' forceps (Dumont #5)

Method

Two different procedures are described for the isolation and handling of GVs, either in aqueous solutions or in mineral oil. See the Discussion for the applicability of the two procedures.

Isolation and observation of GV contents in aqueous spreads

1. Manually dissect individual oocytes from ovary fragments in MBS using watchmakers' forceps. All manipulations and solutions should be at room temperature, 18-20°C.

   Removal of the oocyte follicle, either manually or by collagenase treatment should also be carried out if the microinjection of expression constructs prior to GV isolation is planned.

2. Size-select the separated oocytes according to the stage of oocyte development and optimal chromatin decondensation. Pick oocytes in late stage IV to early stage V (i.e. about 0.8 to 1.1 mm in diameter). Let separated oocytes recover

overnight in MBS before GV isolation to allow re-extension of lampbrush loops and the identification of unhealthy oocytes.

To express fluorescent protein fusions use standard procedures to inject 2-20 ng of synthetic capped RNA into the cytoplasm of each oocyte 24-48 hr before GV isolation.

3. Fill a dispersal/observation chamber with sufficient dispersal medium to form a slightly convex meniscus (Fig. 1D).
4. Using a standard Pasteur pipette transfer several oocytes to a 35 mm Petri dish containing GV isolation medium under a stereo microscope. Use lateral illumination from the light source and a black background. With two pairs of sharp watchmakers' forceps tear an oocyte apart from the vegetal towards the animal pole. The spherical GV sits in the animal hemisphere and can often be seen as a partial clearing in the mass of yolk. Immediately suck the GV into the capillary section of a stretched Pasteur pipette pre-filled with GV isolation medium and transfer the GV swiftly (within 10-20 sec) and with minimal medium or yolk into a dish pre-filled with GV dispersal medium.

   In all GV transfers avoid the presence of air bubbles and sharp or broken edges to the pipette to prevent premature rupture of the GV.
5. Immediately after transferring the GV pick it up with a pair of watchmakers' forceps so as to create a firm grip but not puncture the nuclear envelope. Holding the GV just above the surface of the dish, tear open the nuclear envelope 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. Hold the punctured GV perfectly still in the forceps until the gel-like GV contents spill out of the envelope.
6. Working quickly so that the GV contents do not become too liquid, use a stretched Pasteur pipette pre-filled with GV dispersal medium to separate the

GV contents from the envelope and transfer them in a small volume to the prefilled dispersal/observation chamber from Step 3. The GV contents should sink swiftly to the floor of the chamber.

7. Place a coverslip on top of the chamber. Monitor the extent of dispersal of GV contents in phase contrast with an inverted microscope.

    After 20 min to 1 hr in the dispersal medium the gel should have completely liquefied so that lampbrush chromosomes and GV bodies lie flat on the coverslip forming the chamber floor.

8. When GV contents are fully dispersed, blot excess medium from around the top coverslip with filter paper and seal the edges with molten Vaseline. Detailed observation of unfixed GV structures can be undertaken immediately by phase contrast or DIC microscopy. Likewise, fusion proteins can be immediately monitored by fluorescence microscopy (Fig 1A, B). Spread preparations can be kept for several days at 4°C.

    To more firmly attach the GV contents and to help place most of the chromosome loops in the same focal plane, the preparation can be centrifuged (descriptions of centrifuge adapters for dispersal chambers and centrifugation conditions are described in Morgan 2008).

Isolation and analysis of intact GVs in oil

9. Prepare oocytes as described in Steps 1-2. Transfer a few oocytes in a drop of MBS to a stack of several pieces of filter paper cut to about 1.0 x 0.5 cm. As soon as most of the liquid has drained from the oocytes take the top piece of paper and submerge it and the attached oocytes in 4-5 ml of mineral oil in a 35 mm Petri dish.
10. Orient the oocytes so the animal pole is uppermost and make a small puncture/slash in the top with a sharp needle (e.g. 25G syringe needle).

Depending on the viscosity of the cytoplasm, after a few seconds the GV may begin to emerge spontaneously from the mass of yolk; if not, gently squeeze the oocyte with forceps to encourage the emergence of the GV. With a pipetting device set to 5-8 µl gently suck the GV together with some clean oil into the pipette tip. The GV may be coated with a certain amount of yolk, and some gentle aspiration of the GV in and out of the pipette tip may displace it. Even if some yolk platelets remain attached, transfer the GV to a slide and mount in the oil by gently lowering a coverslip in place.

If studying nuclear bodies use a clean slide. If studying the more delicate chromosomes, use a slide with a circular dam of the wax/vaseline mixture about 50 µm high to surround the GV (Patel et al. 2008).

11. Examine immediately after preparation. Phase contrast or DIC microscopy through yolk-free regions of the intact GV will reveal nuclear bodies at low contrast, while specific targeting of fluorescent fusion proteins to nuclear bodies or chromatin is more striking (Fig 1C, C´).

GVs retain normal physiological activity at room temperature for at least several hours after isolation in oil (Paine et al. 1992).

DISCUSSION

The two procedures described here provide rapid and straightforward means to assess the targeting of fluorescently-labelled proteins in transcriptionally-active chromatin and nuclear bodies at high levels of morphological detail (see Fig. 1). The first procedure provides aqueous spread preparations of nuclear structures in an unfixed though non-functional state and with a massively-diluted nucleoplasmic background. These spreads provide a clear appreciation of the number and location of structures targeted by fluorescent protein fusions in the context of the entire *Xenopus* nuclear genome and its

nuclear bodies. Where use of fluorescent protein fusions is not appropriate/possible, detailed molecular characterization of spread GV contents using immunofluorescence and *in situ* hybridization is also well established. However, additional steps, including centrifugation, are required to produce more permanent, fixed preparations on microscope slides; detailed instructions and advice for these procedures are provided in Gall and Wu 2010 and Gall and Nizami 2016. The second procedure for isolating and maintaining intact GVs in mineral oil enables the analysis of physiologically active nuclei. This procedure was first employed to study molecular dynamics of nuclear bodies (Handwerger et al. 2003; Deryusheva and Gall 2004) and was then adapted (Patel et al. 2008; Austin et al. 2009) to provide a direct visual approach for examining transcription loops and chromatin in real time. Additionally, oil-based manual GV isolation can be used to obtain nuclear material for biochemical investigations (Sommerville 2010).

RECIPES

GV dispersal medium: GV isolation medium (see below) diluted to 25%, $MgCl_2$ adjusted to 1.0 mM overall, 0.1% paraformaldehyde (from 20% stock). Final pH 6.6-6.8 adjusted, with 100 mM KH_2PO_4.

Stored at 4°C.

Just before use add DTT to 1 mM (from 1.0 M frozen stock) and filter through 0.45 μm nitrocellulose.

GV isolation medium: 83 mM KCl, 17 mM NaCl, 6.5 mM Na_2HPO_4, 3.5 mM KH_2PO_4, 1 mM $MgCl_2$. Check final pH 6.9 -7.0.

Store at 4°C.

Just before use add DTT to 1 mM and filter through 0.45 μm nitrocellulose.

Modified Barth's/GTP Saline (MBS): 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5.0 mM HEPES, 2.5 mM pyruvic acid, 0.5 mM theophylline. Final pH 7.5 adjusted with NaOH. Autoclave and store at room temperature. Before use add gentamycin to 50 μg/ml.

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FIGURE LEGEND

Figure 1. (A) - (C) Targeting of fluorescent proteins in Xenopus GV preparations and (D) diagram of a dispersal/observation chamber.

(A) Aqueous spread preparation showing widespread targeting of an RNA-binding protein fused to mCherry both to transcription loops of a lampbrush chromosome and to a type of nuclear body corresponding to somatic cell splicing speckles (examples indicated by arrowheads). Gray-scale fluorescent image.

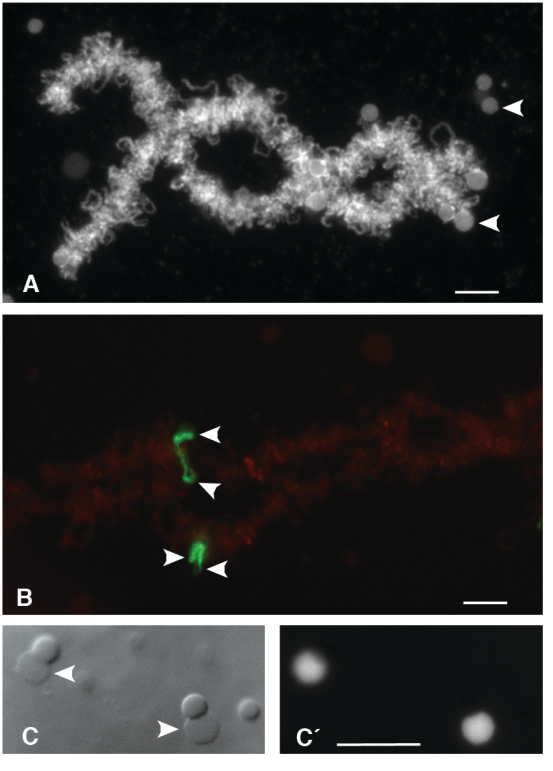
(B) Aqueous spread showing specific targeting of another RNA-binding protein fused to GFP (green) and contrasted with the low-level chromosomal fluorescence provided by a generally-localised protein fused to mCherry (red). The four transcription loops corresponding to a single lampbrush chromosome locus targeted by the GFP fusion are indicated by arrowheads. Combined pseudocoloured fluorescent images.

(C) Nuclear bodies in an intact GV prepared in oil and observed by DIC microscopy. The two objects indicated by arrowheads are histone locus bodies (HLBs) while those attached or adjacent to them are splicing speckles.

(C') Gray-scale fluorescent image showing the specific targeting of a GFP fusion protein to these HLBs.

All scale bars = 10 μm.

(D) Views in section and plan of a chamber constructed from a standard microscope slide that has had a 6 mm diameter hole bored in its centre. The main components of a pre-filled chamber are labelled in the section view.


A
B
C
C´

D