Preparation of lampbrush chromosomes dissected from avian and reptilian growing oocytes

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Abstract

Lampbrush chromosomes represent transcriptionally active chromosomes that exist during the extended diplotene phase in the growing ovarian eggs of many vertebrate animals, except therian mammals. They are excellent for studying chromosome structure and aspects of RNA transcription. Because of their giant size they allow a great increase in the resolution for physical gene mapping. Lampbrush chromosomes have no connection with the nuclear envelope, and can be manually dissected from living oocyte nuclei. Here we present the complete protocol for lampbrush chromosome microsurgical isolation from avian and reptilian germinal vesicles. These chromosome preparations can be further used for fluorescence *in situ* hybridization (FISH) and immunostaining.

Keywords: nucleus, germinal vesicle, lampbrush chromosomes, meiotic chromosomes, microsurgical isolation, Aves, Reptilia

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Introduction

Lampbrush chromosomes are chromosomes of a specific form which appear in growing oocyte nuclei (germinal vesicles) of many animals, including birds and reptiles, due to a highly intense transcription of a variety of sequences. They acquired this name in 1892 because of their extremely large size and a formal resemblance to the brush for cleaning kerosene lamp glass (Rückert 1892). The cause of such similarity lies in the high degree of chromatin despiralization and the presence of numerous lateral loops of DNA, which are extended from chromomeres along the chromosome axis and actively transcribed by RNA polymerase II (Callan 1986). The high degree of decondensation, combined with the preservation of chromosome axial structure, have made lampbrush chromosomes a unique tool for high-resolution gene mapping and genome assembly data verification (Diaz and Gall 1985; Ogawa et al., 1997; Andreozzi et al., 2001; Galkina et al., 2006; Solinhac et al., 2010; Zlotina et al., 2010; Bellott et al., 2017). The first attempts of manual microdissection of lampbrush chromosomes from avian oocyte nuclei were made in the 1960s (Koecke and Muller 1965; Gaginskaya and Gruzova 1969; Ahmad 1970). Although these attempts were based on the technique previously developed for amphibians (Gall 1954; Callan and Lloyd 1960; Macgregor and Varley, 1983), in the area of avian lampbrush chromosome manipulations, the team of the Laboratory of Chromosome Structure and Function of Saint Petersburg State University definitely takes world precedence (Macgregor, 2012). Systematic studies of lampbrush chromosomes from the nuclei of bird oocytes began after publications by Ekaterina Kropotova and Elena Gaginskaya (1984), and by Nancy Hutchison (Hutchinson 1987), where methods of lampbrush chromosome manipulations and preparation for both light and electron microscopy were adapted to the avian lampbrush chromosomes. Irina Solovei, Elena

Gaginskaya, and Herbert Macgregor made a notable contribution to the development of this method (Solovei et al., 1992; 1993; 1994; 1995). Over the last years, however, the main protocol underwent some revisions and improvements aimed at obtaining high quality avian and reptilian lampbrush chromosome preparations that would be suitable for further analysis using immunocytochemistry and fluorescence *in situ* hybridization (FISH).

1. Equipment and instruments

- *Stereo microscope* with bottom lighting and variable intensity control, total magnification from 10x to 100x and min. focal length of 60 mm.
- *Cytological centrifuge* with cooling, min. 3500 g acceleration, with a batch rotor and baskets with lids, allowing horizontal placement of 76×24 mm preparation slides.
- *6V DC power supply* equipped with cleats.
- Stainless steel *ophthalmic scissors.*
- 115 mm histological f*orceps.*
- *Variable volume micropipettes* (up to 20 µl and up to 200 µl) with polypropylene sterile tips.
- *Variable volume micropipettes* (up to 10 µl) with tips made of transparent low-adhesion polypropylene or pipette-stripper with replaceable glass tips with a 150–200 µm in diameter with rounded edges (optional).
- *Glass micropipettes* that can be made from sterile Pasteur glass pipettes by stretching the tip after heating in a burner flame (optional). Depending on the strength and speed of stretching, blanks of different length and thickness can be obtained. With pre-annealed forceps the blanks are broken precisely orthogonally at the level where the diameter of the glass tube is slightly larger than required. The tip of the pipette is placed into the burner flame for melting and smoothing. A small pipette bulb is placed on the wide end of the pipette.
- *Tungsten needles* are used for isolation of oocyte nucleus and removal of nuclear envelope and made of tungsten wire by electrolytic grinding. A wire of 120–500 microns in diameter is cut into 5–7 cm blanks. Then a platinum cathode is connected to the DC source (6V) and immersed into a glass with freshly prepared 10% KOH solution. Tungsten blanks are connected to the positive pole of the current source with an electric clamp and immersed into a beaker with electrolyte. In order to obtain the desired shape of the needle tip, the blank should be immersed into the solution gradually for smooth diameter reduction. As appropriate, the needles can be made of any desired shape (most often, straight, slightly curved and L-shaped tungsten needles are used). The tips of the needles are sharpened additionally by brief contact with the surface of electrolyte solution during operation. This process is controlled under a stereomicroscope. Such needles can be gripped in collet holders. Tungsten tools are sterilized in the flame of the burner.
- *Transparent embryo dish* with 1–2 ml cavity. Before use, the embryo dishes should be washed in soap solution and rinsed with distilled water.
- 76×24 mm *slides,* thickness up to 1.2 mm. The slides are washed in 7X detergent solution (7X or 7X-O-Matic) followed by rinsing with plenty of distilled water. The slides are dried in a thermostat at +60 °C.
- 22×22 mm borosilicate glass *chambers,* 1.2 mm thick, with 1 to 4.4 mm holes. The chambers are pretreated in 70% ethanol and wiped dry before use. The chambers are fixed onto the slide plates strictly in the center with natural rubber glue and left to dry for at least 16 hours at room temperature. The prepared chambers can be stored at +4 °C. The glue layer quality is inspected immediately before use. Excess glue is removed from chamber holes, and holes are filled with hypotonic solution for chromosome isolation.
- *Glass Petri dishes* for preserving the chamber slides from drying during chromosome spreading at +4 °C. Dry filter paper is placed at the bottom of the dish to prevent condensate.
- *A glass container* with a ground stopper for ovary storage and prevention from dehydration during the work process. A sheet of filter paper impregnated with buffer solution is placed at the bottom of the container for this purpose.
- *Saline solution coolant* in a plastic bag, min. size 15×15 cm. The bag with the coolant should be pre-frozen at –20 °C and covered with paper tissue during the work process to prevent the preparation slides from freezing to it.
- 75 cm tall 50 ml *glass beakers* and disposable plastic dishes for solution storage and fixation of the prepared slides.
- *Diamond pencil* for marking slides.

2. Solutions

- *Medium for nucleus isolation «5:1»*: 83 mM KCl, 17 mM NaCl, 6.5 mM Na_2HPO_4, 3.5 mM NaH_2PO_4 (KH_2PO_4), pH 7,0.
- *Hypotonic medium for chromosome isolation «1:4»*: *«5:1»* medium diluted 4 times and containing additionally 0.25 mM $MgCl_2$, 0.1% formaldehyde.
- *Fixative solution: «1:4» medium with* addition of 2% formaldehyde. For immunocytochemistry add 0.25 mM CaCl2; 0.25 mM MgCl2. PBS (phosphate buffered saline) with 2% formaldehyde also can be used.
- Fresh *ethanol solutions* for slide dehydration: 50%, 70% and 96%.

3. Protocol

All manipulations related to lampbrush chromosome isolation should be conducted without heating the solutions and slides, empty embryo dishes and chamber slides must constantly be kept on a cooling surface. The glass contain-

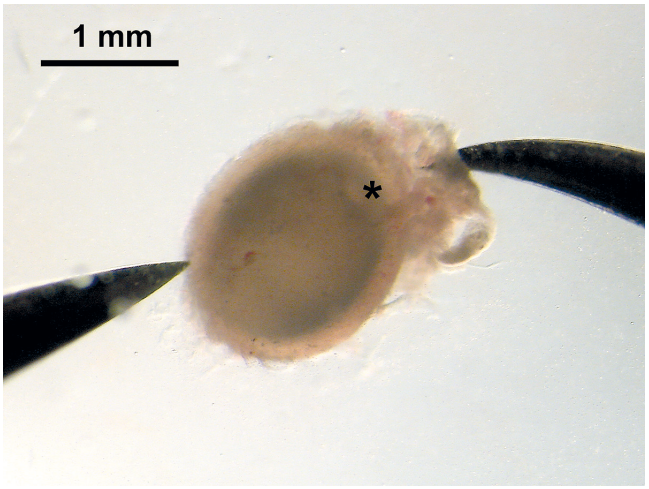

Fig. 1. Cutting an oocyte at the stage of intense growth out of a chicken ovary. Its diameter will be nearly 2 mm, including the tightly fitting follicular epithelium envelope. The area of nucleus location is marked with an asterisk. Hold the oocyte with one needle while the first dissection is being made with the other needle. Then use it to widen the opening and release the content from the follicular membrane

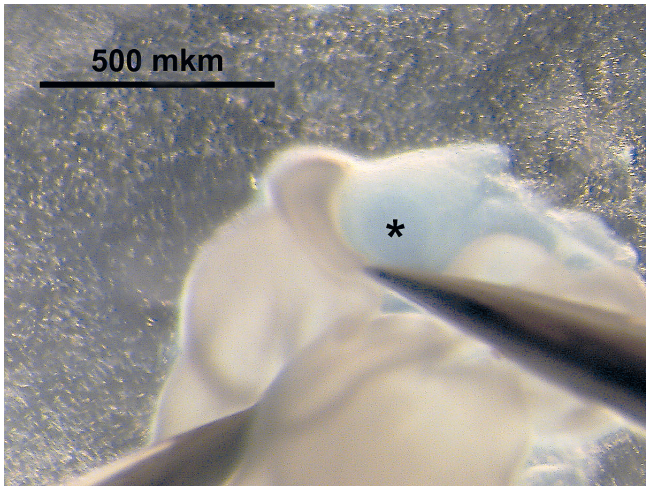

Fig. 2. Chicken oocyte content with a nucleus in it (marked with asterisk). Carefully remove any excess ooplasm (yolk) with the aid of needles and gently release the core. While doing it, avoid needle tip contact with the nuclear surface

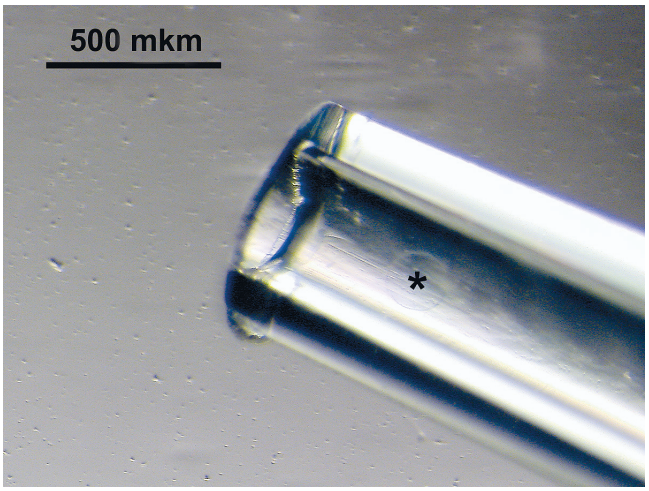

Fig. 3. Oocyte nucleus cleared of excess ooplasm (marked with an asterisk) during transfer into a prepared chamber slide

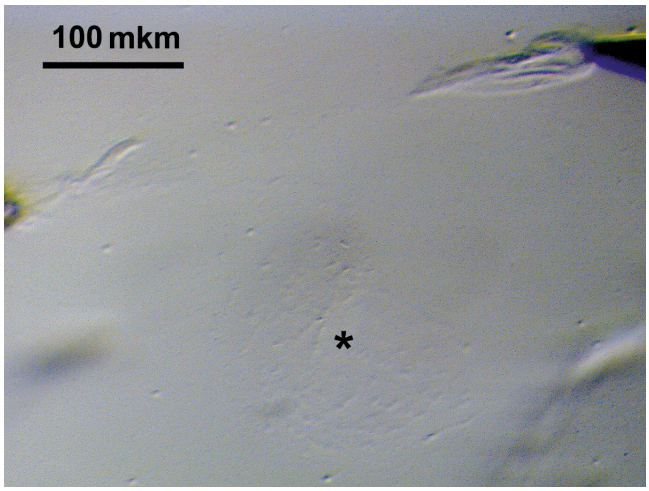

Fig. 4. The nuclear envelope being removed with the aid of thin needles. Karyoplasm with chromosomes sediments to the bottom of the chamber (marked with an asterisk)

er with recently dissected ovary and preparations must be kept in the fridge at +4 °C during spreading. All oocytes in the ovary are surrounded by follicular epithelium and located in the cortical layer of the ovary, the largest of them protruding from its surface. Oocytes of 0.3–1.5 mm in diameter are cut out of the ovary with scissors and put into an embryo dish containing chilled «5 : 1» medium. The position of the nucleus can be identified under a stereomicroscope as a bright area inside the oocyte. Then the oocyte should be arranged in a manner preventing nucleus damage during follicle dissection (Fig. 1).

With two sharp tungsten needles, tear the follicular cover and the plasma membrane of the oocyte and release the nucleus from the ooplasm (Fig. 2). The intact nucleus is completely transparent, yet clearly visible due to the opalescent nuclear envelope, and has a regular spherical shape. Transfer the nucleus into another embryo dish containing hypotonic «1 : 4» medium, and try to remove any ooplasm particles by pipetting the nucleus in and out. Then transfer the nucleus with the same pipette into a chamber slide filled with «1 : 4» medium (Fig. 3). Using thin tungsten needles, remove the nuclear envelope and leave the chromosomes to spread at the bottom of the chamber under the effect of hypotonic solution and gravitational force (Fig. 4).

Keep the chambers containing dissected oocyte nucleus in a Petri dish at +4 °C for 30 minutes, then centrifuge them for 30 minutes at 3500g in a cold (+4 °C) cytological centrifuge. Fix chromosome slides in 2% formaldehyde solution for 30 minutes at room temperature. Dehydrate the preparations in 50 % and 70 % ethanol for 5 minutes

per solution, and then in another portion of 70 % ethanol overnight. The next day, mark the positions of the chamber holes with a diamond pencil on the bottom surface of the slide. Peel the chambers off the slide with the aid of a razor blade, transfer the chromosome preparations into 96 % ethanol for 5 minutes and then air-dry. Dried slides can be stored at room temperature for several months. When preparing lampbrush chromosomes for further immunocytochemical studies, add bivalent metal salts (0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$) to «1 : 4» medium and fixative for preserving the chromatin ribonucleoprotein complexes. In this case, the preparations should be kept in 70 % ethanol at +4 °C without drying and used within a week.

Conclusion

Lampbrush chromosomes from growing oocytes represent a good model system for the analysis of chromosome structure, transcription and processing events with the highest resolution. Nevertheless, contrary to amphibian oocytes the lampbrush chromosome dissection from avian germinal vesicles has been found to have certain difficulties. These difficulties are caused by the follicular envelope, which we are unable to remove without damaging the oocyte membrane because of numerous protrusions of follicular cells into the ooplasm (transosome formation). As a result, it is impossible to conduct a prolonged incubation of living oocyte. It is now possible to find a way to overcome this problem by developing methods of follicular epithelium removal and solutions for released oocyte maintenance.

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