Chapter 17

Counting Chromosomes in Intact Eggs

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Abstract

Chromosomal spreads are an established method to assess ploidy in different cell types. However, many traditional chromosome-spreading techniques require dissolution of the cell and can only be used to assess hyperploidy because of potential chromosome loss inherent in the procedure. Here we describe a method to evaluate chromosome numbers in intact eggs so that both hyperploidy and hypoploidy can be accurately detected.

Key words: Chromosome spread, Chromosome count, Ploidy, Monastrol, Kinetochores

1. Introduction

Traditional chromosome-spreading techniques used to determine chromosome numbers in cells can only detect hyperploidy due to an intrinsic risk of chromosome loss in the procedure. We developed a method that can accurately detect both hyperploidy and hypoploidy in eggs (1). Using monastrol, a kinesin-5 inhibitor, the bipolar spindle collapses into a monopolar spindle and leaves chromosomes dispersed (Fig. 1) (2). After staining DNA and kinetochores, chromosome numbers can be assessed by counting the number of kinetochores in each egg. Several investigators have successfully used this method (3–5).

2. Materials

All materials are prepared with ultrapure water (e.g., using Milli-Q systems from Millipore, Billerica, MA) unless otherwise noted.

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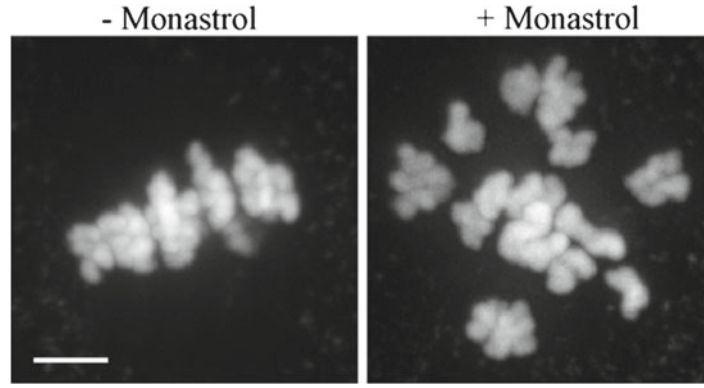

Fig. 1. Monastrol collapses the bipolar spindle and disperses chromosomes. Examples of chromosome configuration in Metaphase II eggs treated either with or without monastrol for 1 h. Images are maximal intensity projections, and scale bar represents 5 μm.

2.1. Oocyte Culture and Maturation

1. CZB maturation medium, modified from ref. 6: 81.62 mM NaCl, 4.83 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, 1.7 mM $CaCl_2$, 25.12 mM $NaHCO_3$, 31.1 mM Na-lactate, 0.27 mM Na-pyruvate, 0.11 mM EDTA, 10 μg/mL Gentamycin, 10 μg/mL Phenol Red, 7 mM Taurine, 3 mg/mL Bovine serum albumin (BSA), and 1 mM L-glutamine (added prior to use). All materials are embryo-culture grade from Sigma-Aldrich (St. Louis, MO).

To make 1 L CZB, measure out 4.77 g NaCl, 360 mg KCl, 160 mg KH_2PO_4, 290 mg $MgSO_4 \cdot 7H_2O$, 250 mg $CaCl_2 \cdot 2H_2O$, 2.11 g $NaHCO_3$, 443 μL Na-lactate (60% syrup), 30 mg Na-pyruvate, 40 mg EDTA-Na_2, 1 mL Gentamycin (10 mg/mL), 1 mL Phenol Red (10 mg/mL), 875 mg Taurine, and 3 g BSA. Add embryo-culture water (Sigma) up to 1 L. Filter-sterilize through 0.22 μm PVDF filters (e.g., Millipore Stericups, Millipore). Store at 4°C.

To prepare 10 mL of 100 mM L-glutamine stock solution, dissolve 146 mg L-glutamine in 10 mL embryo-culture water and filter-sterilize. Store at –20°C.

2.2. Drugs and Fixative

1. Monastrol (Sigma-Aldrich): Prepare stock solution of 100 mM monastrol by dissolving 1 mg monastrol in 34 μL DMSO. Store as instructed.
2. 2% Paraformaldehyde (PFA): Weigh out 0.2 g PFA, add 8 mL water and 100 μL 1 M NaOH (see Note 1). Boil until PFA is dissolved. When solution reaches room temperature, add 500 μL 20× PBS, and adjust pH to 7.4 with 1 M HCl. Bring total volume to 10 mL with water.

2.3. Immunocytochemistry

1. Permeabilization solution: 0.3% BSA, 0.1% Triton X-100, 0.02% NaN_3 in PBS. To make a 500 mL solution, weigh out 1.5 g BSA and 0.1 g NaN_3. Add 25 mL 20× PBS and 500 μL

Triton X-100. Bring total volume to 500 mL with water and filter-sterilize. Store at 4°C.

2. Blocking solution: 0.3% BSA, 0.01% Tween-20, 0.02% NaN_3 in PBS. To make 1 L solution, weigh out 3 g BSA and 0.2 g NaN_3. Add 50 mL 20× PBS and 100 μL Tween-20. Bring total volume to 1 L with water and filter-sterilize. Store at 4°C.
3. Primary antibody: Human antibody against centromeres/CREST (Immunovision, Springdale, AR). Reconstitute with water and store as instructed.
4. Secondary antibody: Alexa fluor 594 goat anti-human (Invitrogen, Carlsbad, CA). Make 2 mg/mL stocks and store as instructed.
5. SYTOX green nucleic acid stain (5 mM in DMSO; Invitrogen). Store as instructed (see Note 2).

3. Methods

1. Add 10 μL stock L-glutamine (100 mM) and 1 μL monastrol (100 mM) to 1 mL CZB, so that the final concentrations are 1 mM and 100 μM, respectively (see Note 3).
2. Pipet 500 μL of CZB + glutamine + monastrol to the inside of an organ culture dish with water in the outer ring (see Note 4). Transfer Metaphase II eggs to center of the dish, with CZB + glutamine + monastrol. Place dish in incubator for 1 h.
3. Prepare 2% PFA and transfer treated eggs to the fixative for 30 min at room temperature.
4. Transfer fixed eggs to Blocking solution (see Note 5).
5. Immunocytochemistry is described below with all solutions in 100 μL volumes in a 96-well dish, but may also be performed in smaller volumes to conserve reagents. Transfer eggs to the well containing 100 μL Permeabilization solution for 15 min, then into 100 μL Blocking solution twice, for 15 min each.
6. To detect kinetochores, transfer eggs into 100 μL Blocking solution containing CREST autoimmune serum (1:40 dilution) and let incubate for 1 h. Wash by transferring eggs through Blocking solution three times, for 15 min each.
7. To detect CREST, transfer into 100 μL Blocking solution containing Alexa fluor 594 goat anti-human secondary antibody (1:100 dilution) and let incubate for 1 h. Wash by transferring eggs through Blocking solution three times, for 15 min each.

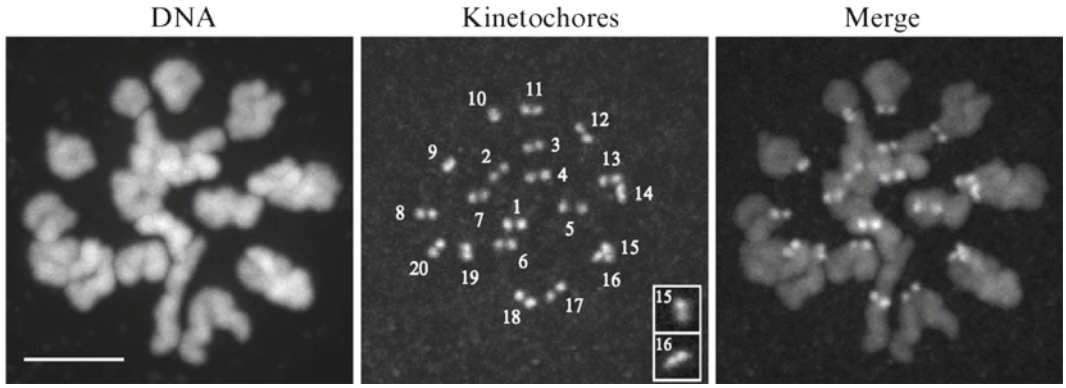

Fig. 2. Assessing ploidy by counting kinetochores. A monastrol-treated Metaphase II egg stained for DNA and kinetochores is shown with numbered kinetochore pairs. Images are maximal intensity projections, with reduced DNA intensity to highlight kinetochores in the merged panel. *Insets* show that kinetochore pairs 15 and 16 are distinct on different z-planes. Note that there are 20 kinetochore pairs in mouse eggs and chromosomes are telocentric. Scale bar represents 5 μm.

8. To stain DNA, transfer eggs to Blocking solution containing SYTOX green nucleic acid stain (1:5,000 dilution) for 10 min.
9. Transfer eggs to mounting medium (e.g., VECTASHIELD, Vector Laboratories, Burlingame, CA) on microscope slides. Image to cover all chromosomes and count the number of kinetochores (Fig. 2) (see Note 6).

4. Notes

1. We find that it is best to prepare this fixative fresh each time.
2. Other stains can be used to detect DNA, such as 4′-6-diamidino-2-phenylindole (DAPI).
3. Prior to use, warm and equilibrate CZB medium in a 37°C incubator with 5-5-90 gas (5% O_2-5% CO_2-90% N_2). Since CZB is not buffered to maintain pH, avoid removing the medium from incubator for extended periods of time; CZB will turn pink in color at high pH. L-glutamine should be added to CZB fresh each time.
4. Other dishes can also be used, but avoid contact with oil if possible since DMSO is oil-soluble.
5. The eggs can be stored overnight at 4°C at this stage.
6. We use a confocal microscope with high magnification (e.g., 100×, NA 1.4). Make sure all chromosomes and especially kinetochores are imaged with fine z-sections (e.g., 0.5 μm intervals).

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