Video Article

A Cell Free Assay to Study Chromatin Decondensation at the End of Mitosis

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Abstract

During the vertebrate cell cycle chromatin undergoes extensive structural and functional changes. Upon mitotic entry, it massively condenses into rod shaped chromosomes which are moved individually by the mitotic spindle apparatus. Mitotic chromatin condensation yields chromosomes compacted fifty-fold denser as in interphase. During exit from mitosis, chromosomes have to re-establish their functional interphase state, which is enclosed by a nuclear envelope and is competent for replication and transcription. The decondensation process is morphologically well described, but in molecular terms poorly understood: We lack knowledge about the underlying molecular events and to a large extent the factors involved as well as their regulation. We describe here a cell-free system that faithfully recapitulates chromatin decondensation *in vitro*, based on mitotic chromatin clusters purified from synchronized HeLa cells and *X. laevis* egg extract. Our cell-free system provides an important tool for further molecular characterization of chromatin decondensation and its co-ordination with processes simultaneously occurring during mitotic exit such as nuclear envelope and pore complex re-assembly.

Video Link

The video component of this article can be found at https://www.jove.com/video/53407/

Introduction

Xenopus laevis egg extract is a powerful and widely applied tool to study complicated cellular events in the simplicity of a cell-free assay. Since their first description by Lohka & Masui [1] they have been extensively used to study mitotic processes such as chromatin condensation [2], spindle assembly [3], nuclear envelope breakdown [4], but also nucleocytoplasmic transport [5] or DNA replication [6]. The events taking place at the end of mitosis, necessary for reformation of the interphasic nucleus such as nuclear envelope reformation and nuclear pore complex reassembly are much less understood compared to the early mitotic events but can be similarly studied using *Xenopus* egg extract [7]. We have recently established an assay based on *Xenopus* egg extract to study chromatin decondensation at the end of mitosis [8], an under-investigated process that awaits its detailed characterization.

In metazoans, chromatin is highly condensed at mitotic entry in order to perform faithfully segregation of the genetic material. To ensure that the chromatin is accessible for gene expression and DNA replication during interphase, it needs to be de-compacted at the end of mitosis. In vertebrates, chromatin is up to fifty-fold more compacted during mitosis compared to interphase [9], in contrast to yeasts where the mitotic compaction is usually much lower, *e.g.,* only two-fold in *S. cerevisiae* [10]. Vertebrate chromatin decondensation has been mostly studied in the context of sperm DNA reorganization after egg fertilization. A molecular mechanism, in which nucleoplasmin, an abundant oocyte protein, exchanges sperm-specific protamines to histones H2A and H2B stored in the egg. This process was also elucidated using *Xenopus* egg extract [11,12]. However, the expression of nucleoplasmin is limited to oocytes [13] and mitotic chromatin does not contain these sperm-specific protamines. Therefore chromatin decondensation at the end of mitosis is nucleoplasmin independent [8].

For the *in vitro* decondensation reaction we employ extract generated from activated *X.laevis* eggs and chromatin clusters isolated from synchronized HeLa cells. Treatment of eggs with a calcium ionophore mimics the calcium release into the oocyte generated by sperm entry during fertilization. The calcium wave triggers the cell cycle resumption and the egg, arrested in the second metaphase of meiosis, progresses to the first interphase [14]. Therefore, egg extracts prepared form activated eggs represent the mitotic exit/interphase state and are competent to induce events specific for mitotic exit like chromatin decondensation, nuclear envelope and pore complex reformation. For the isolation of mitotic chromatin clusters we used a slightly modified version of the protocol published by Gasser & Laemmli [15], where chromosome clusters are released by lysis from HeLa cells synchronized in mitosis and isolated in polyamine containing buffers by gradient centrifugations.

Protocol

Mitotic Chromatin Cluster Isolation from HeLa Cells

1. Preparations

1. **Cell Culture Solutions**
    1. Prepare complete Dulbecco's modified Eagle's medium (DMEM) by adding 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine to the DMEM. Prepare Phosphate buffer saline (PBS) containing 2.7 mM KCl, 137 mM NaCl, 10 mM $Na_2HPO_42H_2O$ and 2 mM KH_2PO_4 in deionized water, and adjust pH to 7.4 with 10 N NaOH.
    NOTE: PBS can be kept as 10x stock solution over time at RT. Dilute it with deionized water to 1x before use. Filter the 1x solution again if it will be used in cell culture.
    2. Prepare a 40 mM stock of thymidine solution (cell culture suitable) in DMEM medium. Dissolve 0.97 g thymidine in 90 ml of DMEM medium. Adjust final volume to 100 ml. Store stock solution at -20 °C. Dissolve (**CAUTION!** work under chemical hood, wear gloves and mouth protection) nocodazole to a 5 mg/ml stock solution in DMSO.

2. **Mitotic Clusters Isolation Solutions**
NOTE: All solutions described in 1.2 need to be kept on ice after preparation/thawing throughout the whole experiment.
    1. Autoclave deionized water for 105 min at 121 °C. Dissolve spermine tetrahydrochloride in autoclaved, deionized water to a final concentration of 200 mM (69.6 mg/ml). Store stock solution at -20 °C. Dissolve spermidine trihydrochloride in autoclaved, deionized water to a final concentration of 200 mM (50.8 mg/ml). Store stock solution at -20 °C.
    2. Prepare 5 % (w/v) digitonin (**CAUTION!** work under chemical hood, wear gloves and mouth protection) in hot, deionized water. Filter and store aliquots at -20 °C. Dissolve phenylmethylsulfonyl fluoride (PMSF) (**CAUTION!** work under chemical hood, wear gloves and mouth protection) to a final concentration of 200 mM (35 mg/ml) in 100% ethanol. Store stock solution at -20 °C.
    3. Dissolve dithiothreitol (DTT) with deionized water to a final concentration of 1 M (154 mg/ml) (**CAUTION!** work under chemical hood, wear gloves). Filter and store stock solution at -20 °C.
    4. Prepare a 100-fold protease inhibitor mix (**CAUTION!** work under chemical hood, wear gloves) by dissolving 10 mg/ml AEBSF (4-(2-Aminoethly-)-benzensulfonylfluoride), 0.2 mg/ml leupeptin, 0.1 mg/ml pepstatin and 0.2 mg/ml aprotinin in deionized water. Store stock solution at -20 °C.
    5. Prepare a 10x stock solution of buffer A containing 150 mM Tris-Cl (pH 7.4), 800 mM KCl, 20 mM EDTA-KOH (pH 7.4), 2 mM spermine tetrahydrochloride and 5 mM spermidine trihydrochloride. Store buffer A at 4 °C without spermine tetrahydrochloride and spermidine trihydrochloride, which should be added freshly just before use.
    NOTE: EDTA only dissolves at pHs higher than 8, therefore, to prepare a high concentrated EDTA-KOH stock solution (0.5 M recommended), add 5 N KOH to pH just above 8 to dissolve it. Afterwards titrate down to pH 7.4.
    6. Prepare a 20x stock solution of buffer As containing 100 mM Tris-HCl (pH 7.4), 400 mM KCl, 400 mM EDTA-KOH (pH 7.4) and 5 mM spermidine trihydrochloride. Buffer As can be stored under same conditions as buffer A.
    NOTE: Prepare the working solutions I to IV (see in the following steps), the glycerol gradient and the colloidal silica particles solutions containing silica particles (15 to 30 nm diameter) coated with non-dialyzable polyvinylpyrrolidone (PVP) freshly just before the isolation procedure (PMSF and digitonin should be added directly before use as PMSF is labile in aqueous solutions and digitonin tends to precipitate upon long term storage on ice).
    7. Prepare 100 ml of solution I by adding 0.5x buffer A, 1 mM DTT, 1:100 of the protease inhibitor mix and 0.1 mM PMSF into autoclaved, deionized water. Prepare 50 ml of solution II (for cell lysis) by adding 1x buffer A, 1 mM DTT, 1:100 of the protease inhibitor mix, 0.1 mM PMSF, 0.1 % digitonin and 10 % glycerol into autoclaved, deionized water.
    8. Prepare 200 ml of solution III containing 0.25x buffer A, 1 mM DTT, 1:100 of the protease inhibitor mix, 0.1 mM PMSF and 0.05 % digitonin in autoclaved, deionized water. Prepare 40 ml of solution IV containing 1x buffer As, 1 mM DTT, 1:100 of the protease inhibitor mix, 0.1 mM PMSF and 0.1 % digitonin in autoclaved, deionized water.
    9. Prepare 120 ml of glycerol gradient solution by adding 25% glycerol and 0.1 % digitonin to solution I.
    10. Prepare 150 ml of colloidal silica particles solution containing 60 % v/v (volume per volume) of a suspension containing silica particles (15 to 30 nm diameter) coated with non-dialyzable polyvinylpyrrolidone (PVP), 15% glycerol, 2 mM spermidine trihydrochloride and 0.8 mM spermine tetrahydrochloride in solution IV.
    11. Prepare cluster storage buffer containing 250 mM sucrose, 15 mM Hepes (pH 7.4), 0.5 mM spermidine trihydrochloride, 0.2 mM spermine tetrahydrochloride, 1:100 of the protease inhibitor mix, 0.3% BSA and 30% glycerol. The cluster storage buffer can be kept at -20 °C.
    12. Prepare squash fix solution containing 10% formaldehyde (**CAUTION!** work under chemical hood, wear gloves), 50% glycerol, twofold Mark's Modified Ringers buffer (MMR see 4.5.) and 0.2 µg/ml DAPI (**CAUTION!** wear gloves). Store at 4 °C in light protected reaction tubes. It is not crucial for the experiment to use this squash fix recipe, alternative recipes will also work.

2. Synchronization of Cells

1. On Day 1: Seed HeLa cells in five 75 cm^2 (250 ml) flasks with media and incubate it at 37 °C in 5% CO_2.
NOTE: This will yield in approximately 18 x 10^6 cells at the day of chromatin cluster isolation.
2. On Day 2: When cells are at least 50% confluent (roughly half of the surface is covered by cells and there is still room for cells to grow), add thymidine to a final concentration of 2 mM (thymidine block) and culture cells for 24 hr at 37 °C in 5% CO_2.

NOTE: This will arrest the cells at the G1/S phase border.
3. On Day 3: Aspirate medium containing thymidine and add sterile PBS. Wash cells by delicate rinsing with sterile PBS. Aspirate PBS and gently add 15-20 ml of fresh, warm complete DMEM medium and culture cells for 3 to 4 hr at 37 °C in 5% CO_2 to release them from the G1/S-phase block.
4. On day 3 (continuation): After releasing the cells from the G1/S-phase block, add nocodazole to a final concentration of 100 ng/ml. Dilute nocodazole by adding 2 µl of stock solution (5 mg/ml) to 98 µl of fresh DMEM medium, and add 1 µl of diluted nocodazole per each ml of cell culture. Culture cells for approximately 12 hr at 37 °C in 5% CO_2. This will block the cells in mitosis.

3. Mitotic Clusters Isolation

1. On day 4: Isolate mitotic clusters. Using a bright field microscopy, check if the majority of cells are mitotic. If less than 50% of the cells are mitotic wait until more cells reach mitosis. Collect mitotic cells by tapping vigorously at the side of the flask (or by gently spraying with the pipette), this will detach remaining mitotic cells. Transfer the cell suspension to 50 ml conical centrifuge tubes.
   NOTE: Mitotic cells become round and can be easily detached from the flask bottom (just like cells after trypsinization), unlike cells in other cell cycle stages, which are flat and firmly attached to the flask.
2. Harvest mitotic cells by spinning the tubes at 1,500 x g for 10 min (4 °C or RT) and removing the supernatant afterwards. Resuspend the cell pellet in 8 ml PBS, pool into one 50 ml conical centrifuge tube, fill the tube completely with PBS and spin again for 10 min at 1,500 x g. Repeat this washing procedure three times in total.
3. From now on perform all steps on ice with cold solutions. Vigorously resuspend the pellet in 37 ml of cold solution II. Transfer the suspension to a cold 40 ml glass-glass homogenizer using a 25 ml pipette and lyse cells on ice by douncing with a tight pestle until mitotic clusters are free of cytoplasmic material. The number of strokes is highly dependent on the digitonin stock and can vary from 3 to 20 times.
   NOTE: Homogenization can be fairly vigorous, but should be considered complete when nearly all mitotic cells are lysed and the clusters are seen to be free of cytoplasmic material (see 3.4).
4. After a couple of strokes mix 5-10 µl of the cell suspension 1:2 with Trypan blue and check by microscopy in a Neubauer chamber. When the cells are lysed chromatin is stained blue and free of cell membranes (NOTE: possible cytoplasmic remnants will be accumulate around the blue stained chromatin and will be easy to distinguish).
   NOTE: Mitotic cells will lyse before interphasic cells but nevertheless be careful not to overdo homogenization in order to avoid contamination with interphasic nuclei and mangled chromatin.
5. Immediately layer the whole cell lysate over cold step gradients (with 5 ml of 60% colloidal silica particles solution at the bottom, overlaid with 19.5 ml of glycerol gradient solution each) in five polycarbonate centrifugation tubes (28.8 x 107.0 mm, it is recommended to place the tubes on ice before to cool them down) using a 10 ml pipette. Do not keep cells in solution II for a long time, thus it is recommended to prepare the tubes and the gradient beforehand (*e.g.,* during the washing steps).
6. Centrifuge the gradients for 30 min at 1,000 x g at 4 °C in a fixed angle rotor.
   NOTE: Nuclei, unlysed cells and clusters are recovered together at the interface of the glycerol and the colloidal silica particles layers.
7. Remove the liquid above the interphase using a pipette and transfer the rest to the cold homogenizer. Re-homogenize mixture by 3-15 strokes (again depending on the digitonin stock) with the tight pestle to eliminate aggregates and to remove cytoskeletal fibers from the clusters. After every couple of strokes check the efficiency of homogenization. Mix 1 µl of the sample with 1 µl of squash fix supplemented with DAPI and examine under the fluorescent microscope.
   NOTE: The number of strokes is crucial, the presence of cluster aggregates means, that the number of strokes is insufficient, while mangled chromatin and nuclei debris indicate that the homogenization was too strong.
8. Distribute the solution among four new polycarbonate centrifugation tubes (28.8 x 107.0 mm) (approx. 10 ml solution per tube) and fill them completely up with 60 % colloidal silica particles solution (approx. 30 ml colloidal silica particles solution per tube).
   NOTE: Avoid overloading the colloidal silica particles gradient since clusters can easily be trapped if there is too much cytoplasmic debris in the gradient.
9. Spin for 5 min at 3,000 x g, followed by 30 min at 45,440 x g at 4 °C in a fixed angle rotor.
   NOTE: As before, interphasic nuclei will be kept from entering the gradient (if homogenization was not done too heavily which releases nuclei from cytoplasmic debris) but the clusters will accumulate around 1.5 cm from the bottom of the tube, often as a loose ball.
10. Remove the liquid above the clusters using a pipette, pool the rest into one tube, resuspend well and redistribute to two polycarbonate centrifugation tubes (28.8 x 107.0 mm). Dilute the cluster suspension 1:4 with solution III in each tube and mix well. Mark the site were the pellet will be and spin 1,000 x g for 15 min at 4 °C in a fixed angle rotor.
11. Resuspend the pellets in Solution III, pool into one 50 ml conical centrifuge tube and fill up with Solution III. Centrifuge at only 300 x g for approximately 10 min. Do not centrifuge at higher velocity - it might cause irreversible aggregation of clusters.
12. Wash again with Solution III in 1.5 or 2 ml reaction tubes (resuspend the pellets and fill the tubes completely up) and centrifuge at 300 x g. Remove the supernatant carefully with a pipette. Resuspend pellet carefully in 250 µl cluster storage buffer (if you have several pellets use 250 µl for all together and pool them). Dilute 5-10 µl of the sample 1:2 with Trypan blue and count in the Neubauer chamber. If applicable dilute more to obtain an approximate concentration of 500 clusters/µl.
13. Push the suspension through a 100 µm cell strainer to make sure to remove cluster aggregations resulting from improper resuspension. The clusters can be stored for months in -80 °C. To avoid multiple refreezing make appropriate aliquots and snap freeze in liquid nitrogen.

4. Preparations of Buffer for Interphasic *Xenopus laevis* Egg Extract

NOTE: *Xenopus laevis* frogs are maintained and treated in accordance with the guidelines and regulations set forth by the Convention of the council of Europe on the protection of vertebrate animals used for experimental and other purposes (EU ratified in 1998) and the German law pertaining to the use of vertebrate animals in research.

1. Prepare DTT and a 100-fold protease inhibitor mix according to 1.2.3 and 1.2.4. Dissolve cytochalasin B to a final concentration of 10 mg/ml in DMSO, aliquot (10 or 20 µl recommended) and store at -20 °C.
2. Dissolve cycloheximide to a final concentration of 20 mg/ml in ethanol, aliquot (500 µl recommended) and store at -20 °C. Dissolve the calcium ionophore A23187 to a final concentration of 2 mg/ml in ethanol, aliquot and store at -20 °C.

NOTE: PI, DTT, cytochalasin B, cycloheximide and A23187 can be repeatedly frozen and thawed.

3. Prepare 20x Mark's Modified Ringers buffer (MMR) containing 2 M NaCl, 40 mM KCl, 20 mM $MgCl_2$, 40 mM $CaCl_2$, 2 mM EDTA and 100 mM Hepes, adjust pH to 8.0 with 5 N KOH.
   NOTE: The 20x MMR can be kept over long time at RT. Depending on the amounts of eggs, for one preparation of interphasic egg extract 1 L of 1x MMR per injected frog and an additional 5-10 liters for the washing steps are necessary. Re-adjust the pH of 1x MMR to 8.0 with 5 N KOH. 1x MMR prepared to keep the frogs in O/N x 1 should be at RT. 1x MMR prepared for the extract preparation should be kept cold until it is used, however it is not crucial for the experiment that the 1x MMR is really cold.
4. Prepare 1 L of sucrose buffer containing 250 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$ and 10 mM Hepes pH 7.5. Sucrose buffer should be prepared the day before using sterile water and should be kept at 4 °C.
5. Prepare the dejellying solution freshly on the morning of the experiment by dissolving 2% L-cystein in 0.25x MMR. Adjust pH to 7.8 with 5 N KOH. Keep at 4 °C until it is used.

5. Protocolfor Interphasic *Xenopus laevis* Egg Extract

1. Inject 120 I.E. pregnant mare's serum gonadotropin (PMSG) into the dorsal lymph sac of each frog 3-10 days before the experiment (5 ml syringes, 27 G ¾'' needles).
   NOTE: This injection will induce ovulation. The amount of eggs one frog lays varies a lot. A well laying frog might produce eggs occupying a volume of up to 7 ml after being de-jellynated which corresponds to up to 3.5 ml of crude extract. However, consider that some frogs might not lay or will lay bad eggs.
2. Inject 500 I.E. human chorionic gonadotropin (hCG) per frog the evening before the experiment (5 ml syringes, 27G ¾'' needles). This will induce the release of the eggs. Keep the frogs for 13-17 hr at 18 °C in individual tanks containing 1.2 l 1x MMR (pH 8).
3. Collect the eggs by pouring them into 600-1,000 ml glass beakers.
   NOTE: Take only the good batches of eggs that are individually laid, similar in size and clearly pigmented with a dark and a light colored half. Do not take eggs that form strings or that look puffy and white. These should be sorted out throughout the whole procedure using a plastic Pasteur pipette. For a detailed description of good versus bad eggs see Gillespie *et al.* [6]
4. Wash eggs intensively, approximately 4 times, with 1x MMR by decanting the supernatant when the eggs have settled down and refilling the beaker with fresh buffer afterwards.
   NOTE: The eggs are stable before they are dejellynated and the washing buffer can be directly applied on the eggs.
5. Dejellynate the eggs by incubation in the 2% cystein solution. Change buffer once after 2-4 min by decanting the buffer and carefully filling the beaker with fresh buffer. Considerdejellying complete when the volume of the eggs drastically decreases and the eggs become more densely packed.
   NOTE: The dejellying needs approximately 5-7 min and should be stopped when visible but latest after 10 min.
6. Wash eggs approximately 4 times with 1x MMR by decanting and refilling the buffer supernatant.
   NOTE: The eggs are more fragile after being dejellynated and, hence, the washing steps need to be done more carefully. The MMR should be rather rinsed on the wall of the beaker instead of directly onto the eggs.
7. Activate eggs in 100 ml 1x MMR by adding 8 µl of the calcium ionophore (2 mg/ml in ethanol). Stop activation when animal cap contraction becomes visible or after 10 min.
   NOTE: The animal cap contraction can be identified by the compaction of the black half of the egg.
8. Wash carefully 4 times with 1x MMR by decanting and refilling the buffer supernatant.
9. Incubate eggs for 20 min in 1x MMR at RT.
10. Prepare the centrifugation tubes during the incubation time: Place 50 µl sucrose buffer, 50 µl 100-fold protease inhibitor mix, 5 µl 1 M DTT , 12.5 µl cycloheximide (to prevent translation, especially of cyclin B) and 2.5 µl cytochalasin B (to prevent actin polymerization) in 5 ml centrifugation tubes (13 x 51 mm). Alternatively, for more than 30 ml of eggs, 14 ml tubes (14 x 95 mm) can be used, in this case increase volumes by 2.4 times.
11. Wash the eggs twice with cold sucrose buffer (decant and refill buffer in the glass beaker) and transfer them into centrifugation tubes using a plastic Pasteur pipette with wide opening (cut off the narrow end).
12. Pack eggs by spinning for 1 min at 130 x g. Put the tubes in 15 ml conical centrifuge tubes for this purpose (put the 14 ml tubes in 50 ml conical centrifuge tubes, respectively). The goal is to remove as much buffer as possible to prevent dilution of the extract. After centrifugation, remove excess of buffer using a plastic Pasteur pipette and eventually fill more eggs on top.
13. Spin in a 6 x 5 ml swing rotor for 20 min at 21,000 x g at 4 °C.
14. Remove low speed extract using a 5 ml syringe with a 16 G 1 ½'' needle, between yellow yolk on top and dark broken egg debris in the bottom. For this purpose, push the syringe needle through the wall of the centrifuge tube just above the layer of broken egg debris in the bottom. Hold the tube against a resistance when pushing with the needle.
   NOTE: A filled 5 ml centrifugation tube gives between 1.8-2.5 ml of extract.
15. Per 1 ml of extract add 10 µl 100-fold protease inhibitor mix, 1 µl of 1 M DTT, 2.5 µl cycloheximide (20 mg/ml) and 0.5 µl cytochalasin B (10 mg/ml). Keep the extract on ice.
   NOTE: The extract can be either used directly for the experiment or aliquoted, snap frozen and stored in liquid nitrogen for several months. Freezing the extract will decrease its activity. For delicate experiments like immunodepletion it is highly recommended to use fresh extract immediately.

6. Preparation of Buffers for *In Vitro* Reconstitution of Chromatin Decondensation

1. Prepare the energy mix stock solution containing 25 mM ATP, 25 mM GTP, 127.5 mg/ml creatine phosphate and 2.5 mg/ml creatine kinase in buffer containing 250 mM sucrose, 1.2 mM Hepes, 5.9 mM KCl and 0.3 mM $MgCl_2$. Aliquot and store at -80 °C. Use freshly after thawing, do not refreeze.
2. Dissolve 0.2 g/ml glycogen in deionized water. Store at -20 °C. Dissolve 6-dimethyl aminopurine (DMAP) to a final concentration of 0.25 M in DMSO. Aliquot and store at -20 °C. Use freshly after thawing, do not refreeze.

3. Prepare 30 % (w/v) sucrose in PBS, filter and store at 4 °C. Prepare 4 % VikiFix solution containing 80 mM PIPES pH 6.8, 1 mM $MgCl_2$, 150 mM sucrose and 4 % paraformaldehyde (PFA) (**CAUTION!** work under chemical hood, wear gloves and mouth protection).
   NOTE: The PFA is difficult to dissolve therefore it is recommended to do it as following: For 1 l Viki-Fix dissolve 24.2 g PIPES and 40 g PFA in separate beakers, both in hot (almost boiling) deionized water. Both will dissolve through addition of 10 N NaOH but be careful to not add too much. Add 51.4 g sucrose and 1 ml 1 M $MgCl_2$ to the PFA solution. Add the PIPES solution to the other mix. Fill up to 1 l final volume and adjust pH to neutral by adding NaOH.
4. Dissolve 10 mg/ml 4',6-diamidino-2-phenylindole (DAPI) in water (**CAUTION!** wear gloves). Store in the dark at -20 °C.

7. Protocol for *In Vitro* Reconstitution of Chromatin Decondensation

1. Spin low speed interphasic extract for 12 min at 386,000 x g in a fixed angle 20 x 0.2 ml or at 355 000 x g in a 10 x 2.0 ml rotor.
2. Gently remove the lipid layer on top using a vacuum pump or pipette and take the supernatant (thereafter called high speed extract) avoiding membrane contamination from the bottom layer and discard the pellet.
   NOTE: To reduce possible membrane contamination it is advisable to spin the extract twice or to dilute the extract with 20 % of the volume with sucrose buffer before the centrifugation. However, dilution and additional centrifugation steps can reduce the extract activity.
3. Pipet 18 µl of high speed extract into a 1.5 ml reaction tube, add 0.7 µl mitotic cluster (amount can be slightly varied according to chromatin stock concentration), 0.5 µl glycogen, 0.5 µl energy mix and 0.3 µl DMAP. Use tips with wide opening to mix the reaction as soon as the chromatin is added to prevent shearing of the decondensing chromatin.
   NOTE: The reaction can be performed in the presence or absence of membranes (see **Figure 3**). To decondense chromatin in the presence of membranes, add 2 µl of floated membranes prepared according to the protocol described by Eisenhardt *et al.* [16]
4. Incubate the reaction mixture for up to 2 hr (or less to study earlier time points of the decondensation process) at 20 °C.
5. Fix the sample by adding ice cold 0.5 ml Viki-Fix containing 0.5% glutaraldehyde and 0.1 mg/ml DAPI and incubation for 20-30 min on ice.
   NOTE: If the samples will be further processed for immunofluorescence, the fixation should be done without glutaraldehyde as this often interferes with the antibody staining. However if only the DAPI staining will be analyzed, the addition of glutaraldehyde will preserve a nicer chromatin structure.
6. Incubate round coverslips (diameter 12 mm) for 5 min with poly-L-lysine solution to increase the affinity of the coverslips to chromatin. Dry the coverslips on filter paper afterwards.
7. Assemble flat-bottom centrifugation tubes (6 ml, 16/55 mm) by putting the coverslips with the coated site to the top on the bottom of the centrifugation tube. Add 800 µl of the 30 % sucrose cushion and layer the fixed sample on top.
8. Spin for 15 min at 2,500 x g at 4 °C.
   NOTE:The flat-bottom centrifugation tubes fit to rotors that adopt 15 ml conical centrifuge tubes.
9. Decant the supernatant, then remove the coverslips from the tubes by poking carefully the bottom of the centrifugation tube with a 16 G 1 ½“ syringe needle. For this purpose tape the lid of the needle and the needle itself together at their bottoms and cut the front end of the lid so that the needle sticks about 3 mm out. When the coverslip is lifted by the needle on one site, use tweezers to remove the coverslip.
10. Wash the coverslip quickly by dipping it in deionized water, dry it gently by touching its side to a filter paper and place it on the microscope slide on a drop of mounting media. Seal it with nail polish, dry and keep in dark.
    NOTE:Samples fixed without glutaraldehyde can be stored in PBS in a 24-well plate and used further for immunofluorescence staining. If stored for several days, add 0.05 % sodium azide (**CAUTION!** wear gloves) to the PBS to avoid contamination with bacteria.
11. Analyze the samples by fluorescence microscopy of the DAPI signal (using *e.g.,* a confocal microscope with a 405 nm laser).

8. Preparation of Buffer for Immunofluorescence Staining of *In Vitro* Reconstituted Chromatin Decondensation Samples

1. Prepare PBS according to 1.1.1. Dissolve NH_4Cl to a final concentration of 50 mM in PBS. Keep this solution at 4 °C. Dissolve 5 µg/ml DAPI in PBS (prepare freshly). Add 0.1 % Triton X-100 to PBS. Keep at 4 °C. Prepare blocking buffer freshly before use by diluting 3 % bovine serum albumin (BSA) in PBS + 0.1 % Triton X-100.

9. Protocol for Immunofluorescence Staining of *In Vitro* Reconstituted Chromatin Decondensation Samples

NOTE: All following incubations of the coverslips are made in a 24-well plate with at least 250 µl solution per well, if not stated otherwise. *In vitro* decondensed chromatin samples are more sensitive than fixed cells therefore be careful when adding or removing solutions. It is recommended to use plastic Pasteur pipettes cut angular. For washing steps and secondary antibody incubation place the plate at RT on rocking or rotating platform, moving not faster than 100 rpm.

1. Quench samples by incubating coverslips with 1 ml NH_4Cl in PBS for 5 min. Block samples by incubating them with 1ml blocking buffer for at least 30 min.
2. Assemble a humidity chamber for the incubation with the primary antibody: Put a wet tissue on the bottom of a closable box and the lid of the 24-well plate upside down on top of the wet tissue. Place parafilm into the lid and add 70 µl of the antibody solution per sample on the parafilm. For the antibody solution, dilute antiserum or affinity purified antibodies 1:100 in blocking buffer.
3. Place the coverslips upside down on top of the antibody solution and incubate them for 1 to 2 hr. Place the coverslips back to the 24-well plate with the sample side facing up and wash samples three times for 10 min with 1 ml 0.1 % Triton X-100 in PBS.
4. Incubate coverslips for 1 hr at RT in 250 µl secondary fluorescent-tagged antibody diluted in blocking buffer to a concentration recommended by the manufacturer. Protect from light. Wash three times for 10 min with 1 ml 0.1 % Triton X-100 in PBS.
5. Incubate the samples for 10 min with 1ml of 5 µg/ml DAPI in PBS. Wash three times for 5 min with 1 ml 0.1 % Triton X-100 in PBS.

6. Wash the coverslip quickly by dipping it in deionized water, dry it gently by touching its side to a filter paper and place it on the microscope slide on top of a drop of mounting media. Seal it with nail polish, dry and keep at 4 °C in the dark until used. Analyze the samples by fluorescence microscopy.

Representative Results

Time dependence of the decondensation reaction

Figure 1 shows a typical time course of the decondensation assay. The cluster of chromosomes visible at the beginning of the reaction decondenses and merges into a single, round and smooth nucleus. When the egg extract is replaced by sucrose buffer the chromosome cluster remains condensed, which suggest that decondensation activity is present in the egg extract.

Chromatin decondensation is an energy dependent process

The *in vitro* decondensation reaction can be conveniently manipulated *e.g.,* by addition of inhibitors. In the experiment shown on **Figure 2**, the non-hydrolyzable ATP or GTP analogs, ATPγS or GTPγS, were added to the reaction. Both inhibit the decondensation showing, that it is an ATP and GTP dependent, active process (**Figure 2**).

Chromatin decondensation and nuclear envelope reformation can be separated

The decondensation assay was performed in the presence or absence of membranes (**Figure 3**). Please note that in both conditions chromatin undergoes decondensation, however addition of membranes results in bigger nuclei. Most probably, reformation of the nuclear envelope induces a secondary decondensation step by yet another mechanism dependent on nuclear transport.

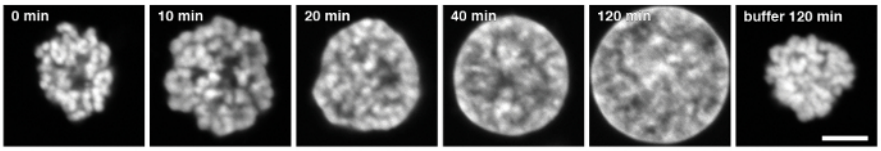

Figure 1. Time course of the *in vitro* decondensation reaction. Mitotic chromatin clusters from HeLa cells were incubated with interphasic *Xenopus* egg extract. Samples were fixed at indicated time points with 4% PFA and 0.5% glutaraldehyde, stained with DAPI and analyzed by confocal microscopy. Re-printed from Magalska *et al.* [8]. Scale bar is 5 µm. Please click here to view a larger version of this figure.

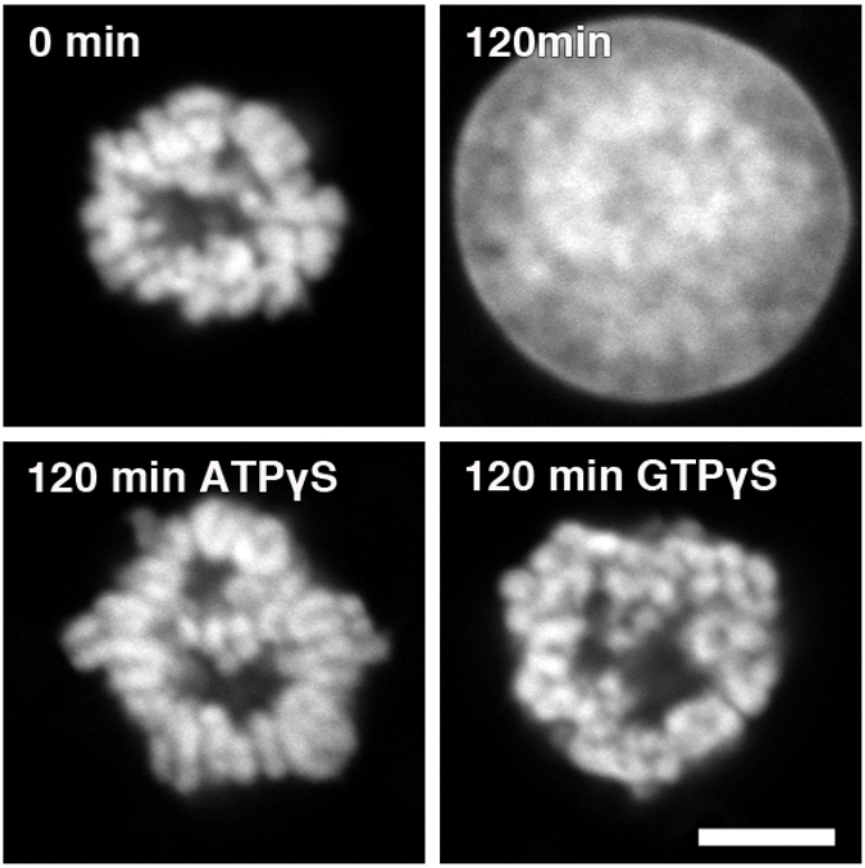

Figure 2. Chromatin decondensation requires ATP and GTP hydrolysis. Chromatin decondensation was performed in the presence of 10 mM ATPγS, 10 mM GTPγS or control buffer. Samples were fixed with 4% PFA and 0.5% glutaraldehyde at indicated time points and analyzed by confocal microscopy. Re-printed from Magalska *et al.* [8]. Scale bar is 5 µm. Please click here to view a larger version of this figure.

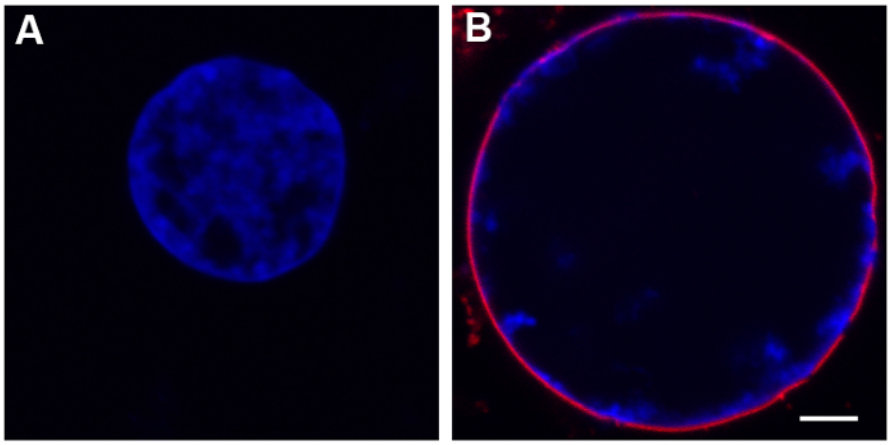

Figure 3. Chromatin decondensation in the presence and absence of membranes. Chromatin decondensation was performed in the absence (**A**) or presence (**B**) of floatation purified membranes for 120 min. Samples were fixed with 4% PFA and 0.5 % glutaraldehyde and analyzed by confocal microscopy. Chromatin is stained with DAPI, membranes with $DiIC_{18}$ (1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate). Scale bar is 5 µm. Please click here to view a larger version of this figure.

Discussion

Xenopus laevis egg extracts are a very useful tool to faithfully reproduce cellular processes *in vitro*, and this system was successfully used in the characterization of cell cycle and cell division events [2,3,5,6,17]. Due to large stores of nuclear components sequestered in the egg during oogenesis, egg extracts are an excellent source of cellular components. Compared to other approaches like RNAi on mammalian tissue cell lines or genetic manipulation, it offers several advantages: The cell-free system allows studying cellular processes in which cellular viability would be otherwise a limitation. Moreover single steps of complex processes can be analyzed in simple assays. The here presented decondensation

assay allows studying molecular mechanisms of postmitotic decondensation with no interference from other mitotic events, respectively. *Xenopus* egg extracts are easy to manipulate by depletion of specific proteins and addition of inhibitors or mutated proteins [8]. For example, **Figure 2** shows the result of adding the non-hydrolyzable ATP or GTP analogs, ATPγS and GTPγS to the decondensation assay. By dilution and differential centrifugation of *Xenopus* eggs components like membranes and cytosol can be separated [16]. **Figure 3** shows the decondensation assay performed in the presence or absence of membranes. Finally, the cell-free assay can also be used to identify novel factors *e.g.,* by a fractionation approach. Using such a strategy we have identified the AAA^+-ATPases RuvBL1/RuvBL2 as crucial decondensation factors [8].

In vitro systems based on *X. laevis* eggs have been employed with different DNA templates: Forbes *et al.* showed that injection of phage λ DNA into unfertilized *X. laevis* eggs induced the assembly of chromatin on naked phage λ DNA. As injection of viral DNA activated the egg, the assembly of chromatin was followed by formation of a nucleus-like structure [18] and similarly λ-phage DNA can be used in combination with egg extracts to generate nucleus like structures *in vitro* [19]. Magnetic beads coated with DNA have been used to study chromatinization of DNA [20] and recruitment of nuclear membranes [21] as well as assembly of a nuclear envelope and pore complexes [22], although it remains open to which extent this resembled a bona fide nuclear re-assembly process. The protocol presented here allows decondensation of isolated mitotic chromatin clusters from HeLa cells using extract generated from activated *Xenopus* eggs. It thoroughly reconstructs events leading to a reformation of an interphasic nucleus [8]. Compared to the widely applied nuclear assembly reaction used to study the formation of the nuclear envelope and the nuclear pore complexes at the end of mitosis, in the decondensation assay HeLa mitotic chromatin clusters instead of sperm DNA are used. Sperm DNA can be assembled into mitotic chromatin or even individual chromosomes upon incubation with extract prepared from unfertilized and non-activated eggs [3]. We decided to use mitotic clusters as chromatin source to simplify the procedure and avoid interference from chromatin condensation. In addition, the preparation of the egg extract is slightly modified: For the chromatin decondensation low speed extract cleared by two high speed centrifugation steps in fixed angle rotors are used. Low speed extract can be stored for up to 6 month in liquid nitrogen without losing its activity. In contrast, in the nuclear assembly reactions, cytosol and floated membranes are generated from low speed extracts by dilution and differential high-speed centrifugation before possible freezing (see Eisenhardt *et al.* [16] for a detailed protocol). In our assay system, addition of membranes allows the formation of a closed nuclear envelope including nuclear pore complexes. The resulting nuclei are competent for nuclear import and export [8]. Thus, this system supports both chromatin decondensation and nuclear envelope reformation. Interestingly, chromatin decondensation is also possible in the absence of membranes (**Figure 3**). However addition of membranes results in slightly bigger nuclei. Most likely, the reformation of the nuclear envelope induces a secondary decondensation step by yet undefined mechanisms, which depends on nuclear import.

For the isolation of mitotic chromatin clusters from HeLa cells, a modified version of the protocol established by Gasser and Laemmli [15] was used. Synchronized mitotic cells are lysed in a buffer containing the non-ionic detergent digitonin and by mechanic forces. The chromatin is isolated as clusters that contain all chromosomes from one nucleus. The crucial difference compared to single chromosome isolation protocols is the fact that the cells are not hypotonically swollen but cooled down to 4 °C before lysis. This prevents the disconnection of the individual chromosomes [15,23]. Compared to the protocol published by J.R. Paulson [23] who recognized the advantage of the isolation of whole chromatin clusters, Gasser & Laemmli used EDTA-containing polyamine buffers instead of Mg^{2+} based buffers to reduce the activity of kinases, nucleases, proteases and phosphatases and by this decrease the amount of protein and DNA modifications occurring during the isolation process [15]. Additionally, using a colloidal silica particles gradient during differential centrifugation highly reduces cytoplasmic contamination. The protocol can also be used to isolate mitotic chromatin clusters from Chinese hamster ovary and mouse cells [15].

Altogether, our protocol faithfully reconstitutes chromatin decondensation as it happens at the end of mitosis. The ATP dependence of the *in vitro* chromatin decondensation can be at least in part explained by the involvement of RuvBL1/2 but also another AAA^+-ATPase, p97, which removes the mitotic kinase Aurora B from the chromatin during mitotic exit [24]. Why the process requires GTP hydrolysis is one of the open questions that we intend to answer using this setup.

Disclosures

The authors have nothing to disclose.

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