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Isolation of Sperm Nuclei and Nuclear Matrices from the Mouse, and Other Rodents

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Summary

The isolation of mammalian sperm heads from their tails is complicated by the relatively high density of the tails, but facilitated by the fact that protamine condensation of the sperm chromatin, and the insolubility of the perinuclear theca make the sperm nucleus stable in SDS. Two methods are described for the isolation of rodent sperm nuclei using sucrose step gradients in which the sperm nuclei are only centrifuged one time, minimizing potential damage by mechanical stress.

Keywords

sperm nuclei; sperm nuclear matrix; sperm nuclear halo; sperm DNA

1. Introduction

The mammalian sperm nucleus is a unique model for the study of chromatin for several reasons. Firstly, the DNA is packaged by proteins found only in that cell type, the protamines (1). Secondly, this nucleus has nuclear matrix that is unusually stable to mechanical stress so that it can be experimentally manipulated with much greater ease than most somatic cell nuclear matrices (2, 3). Thirdly, sperm DNA contains sex specific methylation patterns (4) as well as specific histone modifications (5) that contribute to proper embryogenesis.

The structure of the sperm cell, presents peculiar challenges as well as interesting advantages that make the isolation of the nuclei different from most other cells. The isolation of mammalian sperm heads from their tails is complicated by the relatively high density of the tails which contains the overwhelming majority of the mass of the entire cell (6). This separation is made more difficult by the high density and low viscotic resistance of the tails which makes traditional sucrose density gradients less useful. On the other hand, the high degree of condensation of the sperm DNA by the protamines, and the intermolecular disulfide of the protamines stabilizes the sperm nuclei to such a degree that they can survive sonication (7) and even washing with the ionic detergent sodium dodecyl sulfate (SDS) (8).

The most popular method for isolation of nuclei from cells was developed by Blobel and Potter in 1966 (9). The basis for the isolation was the high sedimentation velocity of nuclei relative to all other components in the somatic cell. Cells were lysed, then centrifuged through a high concentration of cold sucrose resulting in a high yield of nuclei. This method was modified for mouse sperm by adding a discontinuous sucrose gradient with three different concentrations (7). Our longstanding interest in the sperm nucleus has been the organization of DNA by the sperm nuclear matrix. We found that washing spermatozoa with 0.5% SDS was crucial for the isolation of sperm nuclear matrices from hamster (2), mouse (10), and human (11). If sperm nuclei are washed with the non-ionic detergent Triton X-100

(TX100), the sperm nuclei decondense completely when the protamines are extracted. But when the sperm are washed with 0.5% SDS, salt extraction of the protamines results in nuclear matrices that retain the original shape of the sperm nucleus with DNA emanating from the matrices in loops to form a halo around the nucleus.

In this publication, two methods of isolation for the sperm nuclear matrix are presented. The methods were originally developed for isolation of sperm nuclei and nuclear matrices from the hamster, but have since been modified for the mouse. In the first, spermatozoa are washed with SDS immediately upon extraction (SDS Nuclei) (2). The advantage of this method is that nucleases and some proteases are denatured in the first step, possibly protecting the nuclear matrices and DNA. The disadvantage is that the SDS removes the lipid bilayer from the tails, which are not fully dissolved by SDS alone, making them even less boyant in sucrose density gradients and they have a tendency to contaminate the sperm head fraction. In the second method (adapted from (12)), the sperm nuclei are washed with SDS after they are separated from the sperm tails (T-SDS Nuclei).

2. Materials

2.1 – Materials for SDS-Nuclei and Nuclear Matrix

- 1 50 mM Tris-HCL, pH 7.4, 0.5% SDS, 5 mM MgCl₂ (*See Note 1*).
- 2 2 M Sucrose, 50 mM Tris-HCL, pH 7.4 5 mM MgCl₂ (*See Note 2*).
- 3 0.075 g/ml CsCl, 2 M Sucrose 25 mM, Tris-HCL, pH 7.4 (*See Note 3*).

2.2. – Materials for T-SDS-Nuclei

- 4 50 mM Tris-HCL, pH 7.4
- 5 0.45 g/ml CsCl, 25 mM Tris-HCL, pH 7.4, and 0.25 % TX100 (*See Note 4*)
- 6 Sonicator fitted with microtip.

2.3 – Materials Common to Both Methods

- 7 2 M NaCl, 25 mM Tris-HCL, pH 7.4
- 8 1 M dithiothreitol (DTT) stock (*See Note 5*).
- 9 DNase I. (*See Note 6*).
- 10 1 M stock solution of MgCl₂.

¹The MgCl₂ is a holdover from the original Blobel and Potter nuclear isolation method (9). Magnesium condenses chromatin and, consequently, the nuclei, themselves, making them easier to centrifuge through sucrose. This is not required for sperm nuclei. However, another effect of magnesium is to make nuclei less adherent to each other, and this may also be important for sperm nuclei. MgCl₂ is optional in all solutions.

²The 2 M sucrose solutions are difficult to make. The sucrose should be added to a beaker that is larger than the final solution (a 2 liter beaker for a 1 liter solution) with graduations. Then, water should be added to about 90% of the final volume. The sucrose will take up most of the volume. This should be put on a magnetic stirrer with a heater, and the suspension left to stir with the heater set on warm until it is dissolved. The MgCl₂ and the Tris-HCL can be added from 1 M stock solutions. Then the solution can be brought up to the final volume.

³The rationale for the CsCl was to separate the sperm heads from the tails not only by sedimentation velocity through the sucrose but by chemical density as well. The DNA in the sperm heads has a density of 1.7 g/ml compared to that of average protein which is about 1.3 g/ml. The actual concentrations of CsCl in both solutions in these methods were determined by trial and error.

⁴As in Note 3, the rationale for the CsCl was to separate sperm heads based on the density of the DNA they contain. In this solution, the TX100 removes the lipid membrane from the sperm heads as they enter this lowest portion of the step gradient (see Methods), increasing the total density of the sperm heads. Adding the TX100 to this step, rather than an earlier step, allows the tails to retain their lipid membranes, reducing their sedimentation velocity through sucrose.

⁵The 1 M DTT stock solution should be kept in small aliquots in a freezer. The can be thawed several times, but too often results in oxidation.

- 11 Ultracentrifuge with a swing bucket rotor that holds 30 to 40 mls (such as a Beckman SW28).
- 12 100 µg/ml ethidium bromide (EtBr) or DAPI.

3. Methods

3.1 - SDS-Nuclei: Direct SDS Method for Mouse Sperm Nuclear Isolation

- 1 Place the ultracentrifuge rotor in the ultracentrifuge and start the vacuum and refrigeration to 4° C, as described by the manufacturer (*See Note 7*).
- 2 Place the swing buckets on ice and the centrifuge tubes that fit inside them on ice.
- 3 Dissect epididymides from up to 10 mice. Cut them open with scissors and tease out the sperm with small forceps, putting the sperm into 20 ml of 50 mM Tris-HCL, pH 7.4, 0.5% SDS, 5 mM MgCl₂ (*See Note 8*). Mix gently, and place the suspension on ice.
- 4 Set up a triple step gradient as follows in the next three steps. All solutions should be at 4° C. Put 5 mls of 0.075 g/ml CsCl, 2 M Sucrose 25 mM Tris-HCL, pH 7.4, into two ultracentrifuge tubes for a swing bucket rotor with a 35 ml capacity. The centrifuge tubes should be on ice. This is most easily done with a 10 ml syringe (*See Note 9*).
- 5 Carefully overlay 10 mls of 2 M Sucrose, 50 mM Tris-HCL, 5 mM MgCl₂, using a 60 ml syringe to dispense the solution.
- 6 Finally, carefully layer the 20 ml of the sperm suspension onto one of the 10 ml sucrose cushions. This done by tilting the tube, carefully so as not to disturb the sucrose gradient, and slowly pouring the sperm suspension over the sucrose. As the sperm suspension fills, the tube is slowly up-righted. The final triple gradient should have 5 ml of CsCl/sucrose solution at the bottom, with 10 ml of 2 M Sucrose solution over that, with 20 ml of the sperm suspension at the top.
- 7 Centrifuge the triple gradient at 25,000 RPM (113,000 × g) for 1.5 hr at 4° C. Carefully remove the tubes from the swing bucket holders, always keeping the tubes vertical. Occasionally some of the solution spills out over the tube creating suction that requires a small hemostat to help pull them out.
- 8 Aspirate the three layers using a short glass pipette attached to a tube connected to a vacuum source with a large Erlenmeyer flask to collect the aspirate. The sperm heads should be at the bottom (*See Note 10*).

⁶There are two ways to make DNase I stocks. The first is to buy a bovine pancreatic DNase I in solution, and keep it as recommended. A less expensive method is to purchase crystallized DNase I and make a stock solution of 1 mg/ml in 10 mM Tris-HCL, pH 7.4, and freeze it in small aliquots, then thaw the aliquots as needed to a final concentration of 10 mg/ml for the last digestion step.

⁷Most ultracentrifuges require that the vacuum pump be started and the temperature set before placing the rotor with the material to centrifuged inside. The centrifugation will be run at 4° C.

⁸For the mouse, the total amount of sperm nuclei can be increased if one also includes the sperm in the vas deferens. We have found that nucleases exist in the vas deferens fluid that may digest sperm DNA (13), but these are washed away by the procedures that are described (unpublished observations). Make sure to keep mechanical stress low by mixing the epididymal extracts gently with the 50 mM Tris-HCL solution at the start. The SDS will separate the heads from the tails and it is a good idea to make sure this occurred by examining the suspension by phase microscopy. For the hamster, it is possible to use too many sperm. If so, all the heads will be trapped by the tails at the interface. If this happens, repeat with less sperm.

⁹All sucrose solutions in this method should be put into the centrifuge tubes with a syringe rather than a pipette. The solutions are extremely viscous, especially when kept cold, and dispensing them with a syringe is the only method of doing this.

- 9 If the goal is to isolate sperm heads, stop here and resuspend the pellet in the desired solution. If the goal is to make nuclear matrices, or nuclear halos, proceed to step 10.
- 10 Take up the sperm heads with 1 ml of 2 M NaCl, 25 mM Tris-HCL, pH 7.4. Mix gently and avoid a lot of mechanical stress as sperm nuclei are susceptible to this.
- 11 Add 2 mM DTT and incubate at room temperature for 10 to 30 min. Check for nuclear halos by placing 3 μ l of the suspension on a slide, then mixing with 3 μ l of 100 μ g/ml EtBr in 2 M NaCl by gently pipetting up and down. Place a cover slip over the slide and examine under a fluorescent microscope for halos. Fully extracted halos have a bright halo of fluorescence surrounding a nuclear matrix that retains the shape of the sperm head (*See Note 11*). When halos are formed, place the suspension back on ice.
- 12 If the goal is to isolate nuclear halos, stop here. Sperm nuclear halos are very stable and can be centrifuged to remove the salt, but they will clump. An alternative for other procedures, such as restriction endonuclease digestion, is to dilute the sperm halos or even to dialyze the salt.
- 13 If the goal is to isolate sperm nuclear matrices, add DNase I to 50 μ g/ml, and MgCl₂ to 30 mM, and incubate at 37°C for 1 hr. DNase I will digest DNA in 2 M NaCl if the magnesium concentration is high enough. View nuclear matrices under a fluorescent microscope as in Step 11 to ensure that the DNA has been digested. Nuclear matrices will appear hook shaped, as the original sperm heads, but will be very condensed because the DNA has been removed.
- 14 Centrifuge the nuclear matrices at 2,000 RPM ($1,327 \times g$) $\times$ 10 min. Wash with 2 M NaCl 2 $\times$ to remove DNase I, then resuspend in the desired solution.

3.2 – T-SDS Nuceli: Triton X100 then SDS Method for Mouse Sperm Nuclear Isolation

- 15 Set up the rotor and centrifuge tubes as in Steps 1 and 2, above.
- 16 Dissect epididymides from up to 10 mice. Cut them open with scissors and tease out the sperm with small forceps, putting the sperm into 20 ml of 50 mM Tris-HCL, pH 7.4.
- 17 Sonicate the sperm suspension with the mini-probe sonicator at a setting 70% of maximum on ice for 2 mins. This separates the sperm heads and tails and breaks up the tails into smaller fragments. Check the suspension with a phase microscope to ensure that the tails are separated and fragmented. Dilute the suspension with 20 ml 2 M Sucrose, 50 mM Tris-HCL and 5 mM MgCl₂.

¹⁰This aspiration is delicate. Most of the sperm tails will be caught at the interface between the first (50 mM Tris-HCL) and second (2.0 M Sucrose) solutions. Try to aspirate this interface first to avoid contamination of the tails with the sperm heads. Next, try to aspirate the top solution. As you approach the bottom, you may want to leave a small amount of solution so that you do not aspirate the heads. One can often see the nuclear pellet and if it is loose, stop the aspiration before you get to the bottom.

¹¹If the halos are not very large, continue the incubation. If the halos never become large, you can try incubation at 37° C for 5 or 10 min, although this is not usually necessary for mice. Hamster sperm nuclei do require a more elevated temperature. Note that this solution should NOT be viscous even when the halos are fully formed. If the suspension is viscous, it means that the sperm heads have completely decondensed and the nuclear matrices were not stable. Occasionally the sperm heads decondense and lose their original shape usually by the apical ends expanding. This means that the spermatozoa were treated too roughly at the beginning and the preparation is not very good. Less mechanical disruption at the beginning will help with this problem. Also, note that if EtBr is used to visualize the halos, they should appear very small when first seen but grow larger in the 10 to 30 seconds while examining the sperm halos. This is because EtBr supercoils the DNA loops making them small, but in the presence of UV light, EtBr catalyzes single strand breaks in the DNA allowing them to uncoil.

- 18 Set up a triple step gradient as follows: Put 5 mls of 0.45 g/ml CsCl, 25 mM Tris-HCL, pH 7.4, and 0.25 % NP-40 into each SW-28 tube. Carefully overlay 10 mls of 2 M Sucrose, 50 mM Tris-HCL, 5 mM MgCl₂, using a 60 ml syringe to dispense the solution. Finally, carefully layer about 28 ml of the sonicated sperm suspension onto each of the 10 ml sucrose cushions. The final triple gradient should have 5 ml of CsCl solution at the bottom, with 10 ml of 2 M Sucrose solution over that, with 25 ml of the sonicated sperm suspension at the top (*See Note 12*).
- 19 Centrifuge the triple gradient at 20,500 RPM (75,600 × g) for 45 min at 4° C.
- 20 Aspirate the solutions with care to aspirate the broken tails at both interfaces (*See Note 13*).
- 21 If the goal is to isolate sperm nuclei that have not been washed in SDS, the pellet should be resuspend in the final desired solution. If the goal is to make nuclear matrices, continue to Step 22.
- 22 Resuspend the pelleted nuclei in step 5 in 50 ml of 50 mM Tris-HCL, pH 7.4 and spin down at 2,000 RPM × 10 min (This is necessary since even a small amount of CsCl will precipitate the SDS). Resuspend in 50 ml of 50 mM Tris-HCL, pH 7.4 and add 0.5% SDS. Centrifuge at 2,000 rpm × 10 min.
- 23 If the goal is to isolate SDS washed sperm nuclei, stop here. If the goal is to isolate sperm halos or sperm nuclear matrices, continue exactly as described in Steps 11 through 14, above.

Acknowledgments

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¹²The CsCl solution is not viscous at all, and it is sometimes not easy to pour the sucrose onto the top of this solution. An alternative method I have used is to put the 2.0 M sucrose solution in first, then inject the CsCl solution with a syringe that has a small gauge tube attached to the opening underneath the sucrose solution. With either method, the final sperm suspension can be poured on top by gently tilting the tube because the viscous sucrose helps keep the gradient stable.

¹³This aspiration is very different from the one in Step 8 and Note 9. The top and bottom layers are not viscous, and aspirate quickly. The interfaces should be aspirated first, as in Note 9. The pelleted nuclei in this method are much looser, and care must be taken not to aspirate them.

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