Revealing nucleoplasm mechanics by optical trapping and Brownian motion of nucleolus within mouse GV-oocytes in vivo.

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Abstract. Optical trapping of nucleoli within nucleoplasm of living oocytes as unique model system provides non-invasive technique for investigation of nuclear environment. We employed methods of active and passive rheology to characterize rheological properties of the nucleoplasm of GV-oocytes (germinal vesicle stage) with main types of chromatin distribution in the nucleus (NSN, SN). By using of single beam optical trap, formed by a tightly focused laser radiation at 790 nm wavelength, we performed subsequent stress-relaxation tests series of nucleoli in various directions and with different amplitudes. Nucleolus of the oocyte was employed as a microprobe due to its large size and spherical shape. The characteristic nucleolus relaxation times were obtained for two types of chromatin distribution, which can subsequently be used to evaluate the viscoelastic properties of the nuclear material within oocytes with different physiological states. Motion activity of nucleoli was also extracted to evaluate local forces, acting within nucleolar environment and facilitating chromatin redistribution.

1. Introduction
Cells react to extracellular and intracellular signals through structural and functional modifications of nuclear compartments – chromatin and nuclear bodies. Nuclear interior can be described through quantifying key biophysical characteristics of soft living matter – viscosity and elasticity. Biophysics of large-scale chromatin domains and supramolecular complexes correlates with particular physiological states of genome.

Methods of microrheology are designed for probing viscoelastic properties within cells' largest parts (cytoplasm and nucleoplasm) over a wide range of lengths and scales. In order to measure unperturbed Brownian motion of microparticles, embedded into non-equilibrium medium, single- or multiple-particle tracking can be applied. Observation of particle motion provides the opportunity to extract mean-squared displacement (MSD) of the particle, diffusion coefficient, complex shear modulus and to estimate viscoelasticity of the medium. Methods of active rheology require application of external forces on a tracer to induce shear stress within the system. Force is traditionally generated by magnetic field, optical tweezers, atomic force microscope cantilever and micropipette aspiration. [1-3] Varieties of objects can be employed as tracers: vesicles, fluorescent particles, organelles, protein aggregates, quantum dots, magnetic beads etc. Maintenance of integrity and vitality of the cellular interior during rheological measurements is still challenging. Optical trapping represents one of minimally invasive techniques for precise manipulation of nano- or micron-sized objects and generation of forces in the pN range. Minimization of invasiveness is associated with reducing of
heating effect in biological samples due to application of laser radiation within near-infrared spectral (NIR) window of tissue transparency. [4] Optical trapping has proven its effectiveness in studies with different biological objects (viruses, bacterial cells, erythrocytes, organelles, polymer complexes) and processes (cellular adhesion, intercellular contacts, immune response) [5-7].

Damaging potential of chosen technique is critical in situations, when cell is supposed to continue cell cycle or development after measurements. Therefore, reducing of the invasiveness is crucial primarily for germ line cells, especially for oocytes, which serves as reservoirs of maternal factors and structures, determining the direction of embryo development.

In this study, we discuss cellular mechanics in the context of meiosis. Mammalian GV-stage oocytes (GV-germinal vesicle) are characterized by two general types of chromatin distribution within the nucleus – NSN or SN. Nuclei of NSN-oocytes are very similar to interphase nuclei in terms of cell biology due to high rates of synthetic activities and chromatin profile, whereas oocytes of SN-type are characterized by global transcriptional silencing and contain chromatin, condensed around the nucleolus (luminous ring around nucleolus when stained with DNA-specific dye) (Figure 1). [8] Mainly, these oocytes differ in biochemical parameters, morphology, transcriptional activity and developmental competence. Embryos obtained from NSN-type oocytes are believed to stop their development at the 2-cell stage. SN-oocytes produce more advanced stage – blastocyst. [9] Also, intermediate states (partly NSN, partly SN) of chromatin distributions were reported. For today, biochemical and morphological properties defining oocytes’ fate are well studied. Description of GV-oocytes on various levels is of interest for developmental biology and assisted reproductive technology. Combining methods of active and passive rheology and taking into account the differences between chromatin profiles of oocytes with certain physiological states, we tried to extract mechanical properties of their nucleoplasm. Nucleoli of the oocytes served as a tracer particles. To visualize chromatin organization, Hoechst 33342 staining was performed. We tracked motion of nucleolus to probe its motion pattern and MSD. For determination of nucleolus coordinates, software for subdiffraction tracking with lateral resolution up to 6 nm were applied. Optical trapping of nucleoli and series of stress-relaxation tests provided additional information about whole-volume rheological characteristics of nucleoplasm at NSN- or preSN- states of oocytes.

2. Materials and methods

2.1. Collection of oocytes

Female mice (C57BL/6J) aged 6-11 weeks were injected with 7.5 IU PMSG (pregnant mare’s serum gonadotropin). After 48 hours, oocytes were collected from ovaries, placed in M2 medium and then cleaned from cumulus cells with hyaluronidase. Oocytes were transferred into individual 30 μl drop of M2 medium on the coverslip and then on temperature controlled microscope stage. For optical trapping a total of 45 oocytes were examined. Of these, 13 oocytes had NSN-configuration of chromatin, 12 had preSN-SN-configuration. Tracking of internal motion of nucleoli was performed on 30 oocytes (NSN-configuration – 12 oocytes, preSN-SN-configuration – 11 oocytes).

2.2. Laser setup

Optical trapping was performed by continuous wave radiation from a Ti:Sapphire laser (wavelength 790 nm). Laser radiation was coupled to a microscope (Olympus IX71) and was focused by an objective lens (60x 0.7 NA). Laser power at the focus of the objective lens was kept at 280 mW, which corresponds to 10 pN maximal transverse gradient force, applied to a nucleolus in the optical trap and measured previously by calibrated flow in microfluidics system with oocyte. Force of optical trap was calibrated by Stokes’ drag force method.

2.3. Imaging

Nucleoli motion was observed by differential interference contrast microscopy (DIC) and was recorded by high-speed camera at 100 fps (Ximea xiC MC023MG-SY). Videos of thermal motion were being recorded for 2 minutes. Relaxation of nucleoli after displacement by optical trapping was
being recorded until its return into initial position. Oocytes were treated with 1 mg/ml Hoechst 33342 in M2 medium for 10 minutes to identify chromatin distribution.

2.4. Nucleolus tracking
The recorded videos were processed using custom-made software, which provided detection of the nucleolus center coordinates with subpixel and subdiffractional resolution. Mathematical algorithm of coordinates detection was performed through scanning of template across the image with subsequent matching of template with the image for each frame. Resolution was mathematically increased and it was equal 1/16 of the image pixel size or approximately 6 nm. Real resolution distortion due to mechanical vibrations of the setup was estimated by tracking a glass bead fixed on a coverslip and was better than 10 nm. Motion of cytoplasmic membrane was subtracted from nucleolar motion to reduce tracking distortion by movement of oocyte and external vibrations.

2.5. Optical trapping
When the laser was turned on, the nucleolus was pulled into the optical trap and could be moved within the nucleoplasm space. Stress-relaxation tests of the nucleolus were performed sequentially in different directions with an interval of several tens of seconds. Immediately after the relaxation of whole nucleus, chromatin structure was observed (stained with Hoechst 33342). Chromatin visualization was performed after the series of nucleolus displacements to avoid alteration of nucleoplasm viscoelastic response.

2.6. Mathematical analysis
A time-averaged mean square displacement (MSD) as a function of lag time $\tau$ was calculated from the XY-tracks as $\langle \Delta r^2(t) \rangle = \langle (x(t + \tau) - x(t))^2 + (y(t + \tau) - y(t))^2 \rangle$. The average was calculated over times $t$ from 0 to $T - \tau$, where $T$ is the sequence length. Extracted MSD curves for each oocyte was then multiplied by its radius $r$ for normalization and averaged along group ($n=13$ in each). Resulted MSD($r$)*$r$ curves was fitted with power function $A \tau^\alpha$, where $\alpha$ is logarithmic slope, and $A$ is pre-exponential parameter. Exponential fitting of MSD and relaxation curves were performed by OriginPro software (Origin Lab Corporation).

3. Results and discussion
Before all measurements, we sorted oocytes into 2 groups in accordance with chromatin distribution: NSN- or SN-type. (Figure 1). Nuclei of NSN-oocytes are very similar to interphase nuclei in terms of cell biology due to high rates of synthetic activities and chromatin profile. So nucleolus of NSN-oocyte moves throughout nucleoplasm, where chromatin fibers are dispersed and contributes as a dominant force, affecting the nucleolar motion. However, oocytes of SN-type, represent unique situation. In oocytes of SN-type chromatin is condensed around nucleolus, making up one whole body which is subjected to tracking in pure nucleoplasm (without chromatin fibers).

![Figure 1](image_url). Two main profiles of chromatin distribution in oocytes – SN- and NSN-types. (a,c – DIC microscopy; b,d – fluorescent images after Hoechst 33342 staining.)
Diffusion rate and elasticity can be evaluated by power law exponent $\alpha$ of MSD, which can be derived from the logarithmic slope with power function $A\tau^\alpha$ fitting. Averaged MSD($\tau$)$^\alpha$ plot analysis showed that nucleoli of both types of oocytes experience anomalous diffusion (Figure 2). At a small lag times the nucleoli of both oocyte types showed subdiffusive MSD slope and displayed comparable values of about 0.25 (see on Figure 2b,d), describing, however, two distinctive environments. It should be noted that inflection point comes earlier for oocytes of preSN type at $\tau \sim 0.7$ s and $\tau \sim 2$ s for NSN-type, which can be attributed to higher frequency and more active work of motor complexes. At longer lag times (from 10 s), the motion is mainly appearing as superdiffusion with $\alpha > 1$, indicating domination of active transport over diffusion in nucleolar movements.

Figure 2. Tracking of nucleoli motion. (a,c) – trajectories of nucleolar motion for NSN- and partlySN-oocytes and corresponding MSD plots (b,d) with power function fitting.

Optical trapping enabled translocation of nucleoli through the whole volume of nuclei. In oocytes of NSN-type, after displacement of the nucleoli to the maximum distant point we observed strong binding of the nucleolus with nuclear membrane manifested later as abnormally small nucleolus displacements under the maximum power of optical trap applied and its specific deformation. Oocytes of the advanced (and probably terminal) SN-type were characterized by attachment of nucleolus to the nuclear membrane and this type of attachment occurred in several points (i.e. probably mediated by condensation of several chromosomes). Therefore, we employed oocytes of partlySN-stage (with the same chromatin distribution as in oocytes of SN-type, but another mode of nucleolar movements) for tracking. Within oocytes of partlySN-type relatively free nucleolar motion were observed, which can be explained by loosening of nuclear envelope or partly decondensed state of certain chromatin fibers.
(or both). We observed differences between mechanical characteristics of nucleoli on different stages. Nucleoli of NSN-type oocytes were highly vacuolated and, in some cases, undeformable or slightly deformable, whereas nucleoli of partlySN- and SN- type possessed smoother surface and more fluid-like behaviour. (Figure 3d,f).

For tracking of nucleolar motion, small displacements of nucleoli by optical trapping were performed to avoid its own deformations and involvement of cytoskeleton/nuclear envelope deformations into resulting relaxation curves. Furthermore, nucleoli of all oocytes from the group, chosen for curve analysis, were displaced in a same way in order to not confuse displacements of nucleolus with its own deformation. So, in final selection 5 oocytes of each type were included and 13 measurements for each type were extracted. Small displacements revealed different relaxation times between oocytes of NSN and partlySN types. (Figure 3a,b). Curve fitting were best performed on times up to 20 seconds (fast component) due to the fact that in some cases relaxation were interrupted by fluctuations of relatively high amplitude and correct fitting were not possible. (Figure 3c). These fluctuations probably correspond to the molecular motors activity, which have been already observed before as a superdiffusive parts of MSD plots at the same lag times (~10 s) comparable to fluctuation frequency (~0.1 Hz).

![Figure 3](image-url)

**Figure 3.** Nucleolar manipulation with optical trapping and its relaxation curves. (a,b) – representative relaxation curves of individual oocytes of certain categories with qualitative characteristics; (c) - nucleolar fluctuations during relaxation after turning-off the optical trap; (d,f) – patterns of nucleolar motion within oocytes of NSN- and partlySN (corresponds to pSN on the graph)-types respectively.
Relaxation of nucleoli could be fitted by mono- or multi-exponential decay. It emphasizes complexity of interactions between nuclear components within non-equilibrium system. We use biexponential model based on Kelvin–Voigt model as generally optimal. We use fitting equation $r(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$, where $r(t)$ is displacement of nucleolus from equilibrium position, and $\tau_1$ and $\tau_2$ are characteristic relaxation times, defined as $\tau = \eta/E$, where $\eta$ is the viscosity and $E$ is a modulus of elasticity of viscoelastic material. In this model we find averaged relaxation times for SNM oocytes: $\tau_{1SN}=0.69$ s, $\tau_{2SN}=8.58$ s and for partlySN oocytes: $\tau_{1pSN}=2.55$ s, $\tau_{2pSN}=18.1$ s. Different configuration of chromatin distribution in these oocytes not only manifest itself as different relaxation times, but also as different contribution of fast and slow components in overall relaxation kinetics. Value of amplitude $A_1$ for oocytes of partlySN-type was substantially larger than $A_2$, reveal the predominance of fast component over the slow one and indicating on possible lower viscosity of partlySN oocyte nucleoplasm. (Figure 3a,b) An accurate interpretation of results at this point is difficult. Many factors, affecting the movement of the nucleolus, should be taken into account, i.e. influences of chromatin, cytoskeleton, nuclear envelope, nuclear lamina.

4. Conclusions
Nuclear interior and interactions between nuclear compartments of living mice oocytes can be studied by means of optical trapping combined with the analysis of thermal motion with nucleolus as a tracer particle, allowing more extensive research of nuclear interior. MSD plots and relaxation curves after nucleolus displacements showed that at time of several seconds the action of nuclear motor complexes become visible. For two main types of GV-oocytes (SN(partlySN) and SN) different characteristic relaxation times were observed indicating alterations in viscosity/elasticity ratio. These data will be useful for subsequent measurement of complex shear moduli and specifying viscoelastic properties for oocytes of both types. Fluctuations, appeared on relaxation kinetics of nucleolus, have frequency corresponding to superdiffusive parts of MSD curves and demonstrate activity of nuclear motor complexes. Mechanical characteristics of cell nucleus provide the basis for biophysical criteria for assessment oocyte quality and set the link between well studied morphological and biochemical properties of GV-oocytes. Optical trapping of nucleolus opens up possibilities for studying physical properties of the nucleolus despite the specificity of biochemical characteristics and functioning of nucleoli within germ cells.

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