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Report

Anisotropic Diffusion of Macromolecules in the Contiguous Nucleocytoplasmic Fluid during Eukaryotic Cell Division

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Summary

Character and rapidity of protein diffusion in intracellular fluids are key determinants of the dynamics and steady state of a plethora of biochemical reactions [1, 2]. So far, an anomalous diffusion in cytoplasmic fluids with viscoelastic and even glassy characteristics has been reported in a variety of organisms on several length scales and timescales [3–6]. Here, we show that the contiguous fluid of former cytoplasm and nucleoplasm features an anisotropically varying diffusion of macromolecules during eukaryotic cell division. In metaphase, diffusion in the contiguous nucleocytoplasmic fluid appears less anomalous along the spindle axis as compared to perpendicular directions. As a consequence, the long-time diffusion of macromolecules preferentially points along the spindle axis, leading to prolonged residence of macromolecules in the spindle region. Based on our experimental data, we suggest that anisotropic diffusion facilitates the encounter and interaction of spindle-associated proteins, e.g., during the formation of a dynamic spindle matrix [7].

Results and Discussion

Eukaryotes with an open mitosis (e.g., mammalian cells) show major structural rearrangements during the cell cycle [8]. In interphase, the nuclear envelope restricts and controls trafficking of macromolecules between the nucleoplasm and the disjunct exterior cytoplasm. Yet, after nuclear envelope breakdown (NEB) at the onset of mitosis, cytoplasm and nucleoplasm form a contiguous nucleocytoplasmic fluid in which cytosolic macromolecules can freely invade the nuclear region [9]. Due to large amounts of metabolic energy converted in and at the mitotic spindle apparatus, the crowded nucleocytoplasmic fluid is constantly driven out of thermal equilibrium. Moreover, oriented microtubules in the spindle equip this region with an almost-nematic character. The diffusional motion of proteins and other macromolecules in and around the spindle therefore can be expected to show unique and spatially varying features that eventually determine the progress of vital biochemical reactions [1, 2].

To elucidate the properties of the contiguous nucleocytoplasmic fluid during mitosis, we monitored the local diffusion behavior of enhanced green fluorescent protein (EGFP) and fluorescently tagged dextran molecules ("fluo-dextran") in a spatially resolved manner. In particular, we used fluorescence correlation spectroscopy (FCS) as a method to determine the local diffusion characteristics of EGFP and fluo-dextran along and across the spindle apparatus during metaphase (cf. Figures 1A–1C). Both EGFP and fluo-dextran do not interact in a specific fashion with the cell's interior, and, therefore, they are adequate reporter particles for plain diffusion in the nucleocytoplasmic fluid. Given that EGFP and fluo-dextran have diameters of about 1.5 nm and 15 nm [10, 11], respectively, these tracer particles also test the fluid's properties on different length scales: EGFP probes the length scale of the majority of proteins, whereas fluo-dextran is a model for macromolecular complexes or small organelle fragments.

As a result of our experiments, we observed an anisotropically varying behavior of the fluorescence autocorrelation decay for both tracer particles in about 30% of all tested mitotic culture cells that showed a metaphase phenotype (Figures 1A and 1B). Each of these cells exhibited systematic rather than purely stochastic variations of FCS curves between the chosen measurement points. The remaining 70% of the cell ensemble showed a very weak (~20%) or undetectable (~50%) systematic variation of FCS curves even though the cellular phenotype was not different. Most likely, in these cells, unavoidable stochastic variations of FCS curves between the limited set of measurement loci masked the systematic trend (see also Supplemental Experimental Procedures available online). Increasing the number of measurement loci is a potential means to reveal a systematic trend, even for noisy data; yet, in our case, this approach would have also enhanced bleaching, possibly introducing artifacts [12]. We therefore refrained from doing this and focused our analysis on 30% of the cell ensemble that showed a clear trend, bearing in mind that this selection may report an upper bound of the phenomenon.

Our FCS measurements revealed that the mean dwell time, τ_D, of EGFP in the diffraction-limited confocal volume varied systematically, perpendicular to the spindle axis, while little changes were observed along the spindle axis (Figure 1D; see also Figure S1 for representative FCS curves): EGFP stayed about 2-fold longer in the confocal volume when FCS measurements were performed in the spindle center or along the spindle axis as compared to more remote regions perpendicular to the spindle axis. This observation suggests that EGFP's diffusional motion is impeded in the spindle region in an anisotropic fashion, with a higher degree of obstruction along the spindle axis. Given that EGFP acts as an inert tracer, i.e., it does not interact specifically with cellular protein machineries, this observation yields a first indication for anisotropically varying material properties of the nucleocytoplasmic fluid along and across the mitotic spindle. Indeed, these material properties determine the diffusional motion not only of EGFP but also of all macromolecules in the spindle region. We would like to emphasize at this point that the variation of the mean dwell time is only about 2-fold on the cell's length scale ($\lambda \approx 10\ \mu m$), i.e., within the FCS focus, an anisotropy of dwell times along or perpendicular to the spindle axis is at maximum 5%–10% and hence too small to be picked up reliably by FCS. Therefore, the assumption of an isotropic dwell time on these short length scales, i.e., within the FCS focus, is justified, whereas an appreciable anisotropy is seen on larger length scales.

To gain deeper insights into the anisotropically varying diffusion behavior of EGFP in the spindle region, we inspected the diffusion anomaly α that determines the steepness of the

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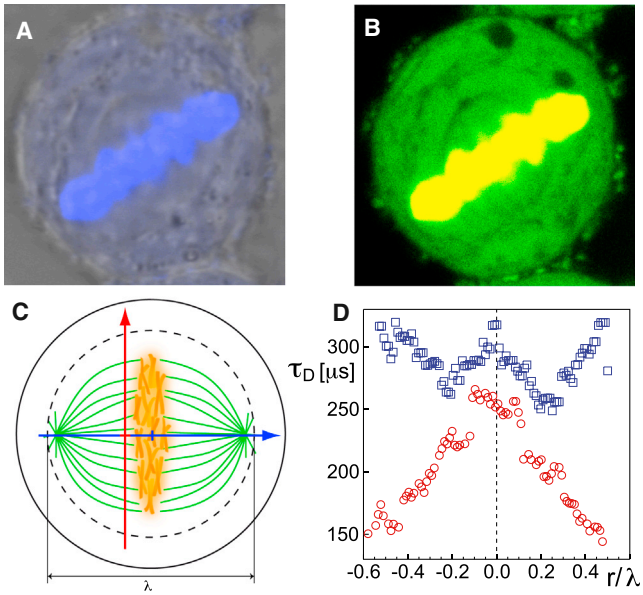

Figure 1. Diffusion Varies Anisotropically in Metaphase Cells

(A) Bright-field image of a HeLa cell in metaphase. DNA staining with fluorescent DAPI (blue) highlights the chromatin-rich metaphase plate in the spindle center.

(B) EGFP's fluorescence (green) in the same cell reveals a diffuse staining of the entire contiguous nucleocytoplasmic fluid (DNA stain superimposed in yellow).

(C) Sketch of a mitotic cell with the spindle apparatus (green) and chromatids (orange) on the metaphase plate. Dashed circle indicates the size of the spindle region (diameter λ); full line indicates the cell's envelope. Red and blue arrows depict directions perpendicular and parallel to the spindle axis along which FCS data were acquired.

(D) Characteristic fluorescence correlation decay time, τ_D, obtained for free EGFP at positions along and perpendicular to the spindle axis in an ensemble of cells (blue squares and red circles, respectively). To gain statistics across the cell ensemble, we rescaled measurement positions r (defined with respect to the cell's spindle center) with the individual spindle dimension λ. To reduce fluctuations, we applied a moving average (window width = 0.1). Although τ_D shows few changes along the spindle axis (blue squares), systematic changes are evident for data taken perpendicular to the spindle axis (red circles). See Results and Discussion for more details.

autocorrelation decay in our FCS curves (cf. equation 1 in Supplemental Experimental Procedures). If EGFP's diffusional motion could be assumed to not be governed by strong nonequilibrium processes, the diffusion anomaly α also would determine the frequency scaling of the nucleocytoplasmic fluid's shear modulus ($G(\omega) = G'(\omega)+iG''(\omega)$), i.e., $|G(\omega)| \sim \omega^{\alpha}$ [4, 13, 14]. Real and imaginary parts, $G'(\omega)$ and $G''(\omega)$, reflect the elastic and viscous characteristics of a fluid that is being sheared with frequency ω, and a frequency-dependent viscosity is given by $\eta(\omega) = G''(\omega)/\omega \sim \omega^{1-\alpha}$. Indeed, cytoplasm and nucleoplasm of interphase cells have been reported to be viscoelastic on several length scales and timescales [3–5], i.e., $G'(\omega)$, $G''(\omega) \neq 0$, due to a scaling exponent $\alpha < 1$. Yet, it is unclear whether we can assume here that passive microrheology, i.e., obtaining $G(\omega)$ from diffusion data, is a valid approach (see below). We therefore show representative curves for the frequency-dependent viscosity only as a supplemental figure (Figure S2), with the caveat that it may lack important nonequilibrium contributions [14] (see also below).

As a result, we observed that the diffusion anomaly α was always smaller than unity at all measurement positions, whereas its actual value varied significantly between different measurement loci (Figure 2A): values of α showed a systematic anisotropic variation similar to our results on the mean dwell times, τ_D (cf. Figure 1D). Indeed, the nucleocytoplasmic fluid showed almost-normal diffusion characteristics ($\alpha \rightarrow 1$) inside and along the spindle, whereas a gradually increasing subdiffusion (α well below unity) was observed perpendicular to the spindle axis when leaving the spindle center. In fact, the observed anomaly values of the nucleocytoplasmic fluid far away from the spindle center compare favorably to previous reports on various length scales and timescales [4, 15].

Thus, the nucleocytoplasmic fluid not only exhibits an increased hindrance of EGFP's diffusional motion along the spindle axis (as evidenced by τ_D) but also appears to feature a less-anomalous diffusion along the spindle as compared to the perpendicular axis, which is somewhat counterintuitive. This observation suggests that molecular diffusion on short and long timescales always has an anisotropic character, yet with a swap of the preferred direction of spreading (see details below).

We next asked to what extent our results for EGFP also hold true for the approximately 10-fold larger fluo-dextran molecules that probe nucleocytoplasmic fluid properties on larger length scales. As expected from the tracer's size, FCS revealed a roughly 10-fold-lower mobility of fluo-dextran as compared to EGFP, i.e., the mean residence time, τ_D, was about 10-fold larger (Figure S3). Moreover, in line with our data on EGFP, we observed again a systematic change of τ_D and the anomaly exponent α perpendicular to the spindle axis, whereas no systematic changes were seen for data taken along the spindle axis (Figure S4). Yet, the size of fluo-dextran and potentially its somewhat harsh incorporation into cells via electroporation also lead to some distinct differences: in the very center of the spindle, we were not able to obtain meaningful FCS curves. Fluo-dextran was immobilized in these loci (e.g., squeezed between cytoskeletal elements of the spindle), and/or it was partially size excluded from this region. In addition, FCS curves (and hence values of τ_D and α) varied stronger at similar measurement loci as compared to our EGFP data. We attribute this to the fact that fluo-dextran probes the complex nucleocytoplasmic fluid and its crowders on larger length scales that are less representative for the majority of proteins in the cell. Yet, despite these details, the data on fluo-dextran are in favorable agreement with our observations for EGFP.

Given our experimental observations, how can one rationalize the anisotropically varying properties of the nucleocytoplasmic fluid? In principle, there are two explanations that are not mutually exclusive for the observed anisotropy in the long-range diffusion. First, a plethora of proteins, e.g., molecular motors [16] and/or members of a spindle matrix [17], converts large amounts of metabolic energy during metaphase, and a temporally varying bidirectional flux of material along the spindle axis has been described [18]. Therefore, the conversion of metabolic energy to a bidirectional flux of material could add a nondirected, ATP/GTP-derived random force to diffusional motion along the spindle axis, whereas diffusional motion in the perpendicular direction is only fed by thermal energy. As a consequence, the macromolecules' random walk along these two directions would differ by a flux-induced, metabolic energy contribution that renders the molecules' random walk truly nonequilibrium. Following this rationale, the bidirectional flux along the spindle axis would fluidize the nucleocytoplasmic liquid inside and along the spindle, whereas the fluid stays strongly viscoelastic in the perpendicular direction. Similar fluidization phenomena, albeit without anisotropy, have been

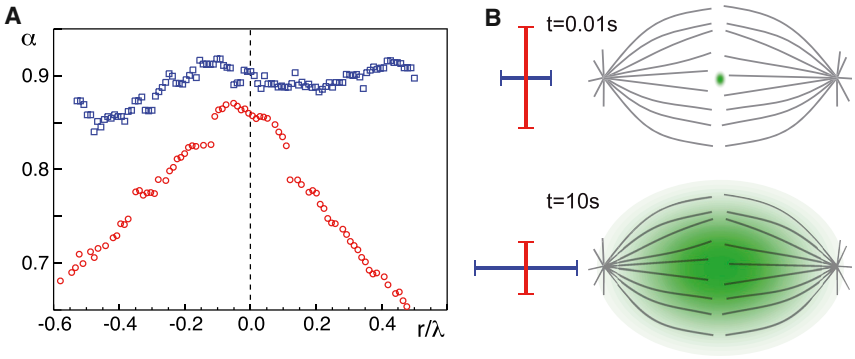

Figure 2. Preferential Diffusion along the Spindle Axis due to Anisotropic Material Properties of the Nucleocytoplasmic Fluid

(A) Scaling exponent α along (blue squares) and perpendicular (red circles) to the spindle axis for the cell ensemble shown in Figure 1D (same smoothing). Whereas diffusion in the nucleocytoplasmic fluid appears almost normal along the spindle on the scale of EGFP (i.e., $\alpha \rightarrow 1$), a significant decrease of the exponent α, highlighting a pronounced diffusion anomaly, is observed perpendicular to the spindle axis.

(B) Simulation snapshots of an ensemble of EGFP molecules spreading diffusively from the spindle center using the experimental data of Figures 1D and 2A (see Results and Discussion for details). Anisotropies are highlighted by the aspect ratio of the vertical red and horizontal blue bars on the left. For short times (t = 10 ms; top), EGFP preferentially explores the direction perpendicular to the spindle because short-time diffusion is mostly dictated by the values of τ_D (note that τ_D is larger along the spindle axis, cf. Figure 1D). For large timescales (t = 10 s; bottom), a prevailing diffusion along the spindle axis is seen while the perpendicular direction is explored to a lower extent. In contrast to the short-time spreading at t = 10 ms, this anisotropic spreading is mainly fuelled by the different exponents α along and perpendicular to the spindle axis (note that α is larger along the spindle axis).

observed very recently in bacteria [6] also, where even a glassy cytoplasmic state was reported in the absence of metabolic energy conversion. Alternatively, a second and putatively more likely phenomenon can be used to rationalize our experimental observation: the spindle's organization naturally breaks symmetry by the presence of oriented microtubules with which a large number of proteins, membrane fragments, etc. are associated. The spindle area therefore appears striated, and diffusion in this environment is reminiscent of a random walk in a nematically ordered liquid, i.e., it is easier to move along filaments rather than to move perpendicular to them. As a consequence, long-range diffusion perpendicular to microtubules would be hindered, whereas diffusion along the spindle axis would be less obstructed.

Our experimental data and the cells' phenotype did not allow us to distinguish between these two possibilities. Yet, at least for the first line of reasoning, the translation of diffusion data into a complex shear modulus is invalid due to the nonequilibrium contributions [14]. An explanation in terms of a nematically ordered spindle region may formally allow for a translation of diffusion data into a complex shear modulus. However, in the extreme case of statically ordered filaments, the diffusive motion of proteins rather resembles a random walk in an anisotropic maze (percolation scenario), and hence the use of passive microrheology again is questionable. If the maze itself is not static but instead has properties of a gel, results obtained by passive microrheology may be a meaningful way to describe the contiguous nucleocytoplasmic fluid.

Regardless of the microscopic causes, the anisotropically varying diffusion properties in the nucleocytoplasmic fluid are likely to have an impact on molecular events in and around the spindle. To explore such consequences, we took the experimental data of Figures 1D and 2A and modeled the molecules' diffusional spreading in x and y direction (along and perpendicular to the spindle axis, respectively) by a Gaussian propagator of the fractional Brownian motion type [1], i.e., $\phi(x.y,t) \sim \exp(-x^2(\tau_D^{(x)}/t)^{\alpha_x} - y^2(\tau_D^{(y)}/t)^{\alpha_y})/t^{(\alpha_x+\alpha_y)/2}$. Here, distances x and y are given in units of the focus size (about 220 nm), and $\tau_D^{(x)} = 300\ \mu s$, $\alpha_x = 0.9$ and $\tau_D^{(y)} = 150\ \mu s$, $\alpha_y = 0.7$ are the experimental results along and perpendicular to the spindle axis, away from the spindle center (cf. Figures 1D and 2A). Please note that this definition of the propagator extrapolates the observed long-range diffusion anisotropy to smaller length scales and timescales, which we could not assess directly with our FCS experiments. Assuming now that a particle starts in the center of the spindle, the diffusion propagator $\phi(x,y,t)$ predicts a preferential diffusion perpendicular to the spindle axis for small times because $\tau_D^{(y)} < \tau_D^{(x)}$ (Figure 2B, top). However, owing to the different anomaly exponents ($\alpha_x > \alpha_y$), a preferential diffusive spreading along the spindle axis is observed for extended periods (Figure 2B, bottom). As a consequence, protein diffusion is effectively bound to the spindle region over a long period, but an effective diffusive mixing within and along the spindle apparatus remains possible. As a consequence of such an effective confinement, formation of interacting networks of spindle-associated proteins may be facilitated because participating molecules meet each other more frequently than they would via isotropic diffusion. The anisotropic properties of the nucleocytoplasmic fluid therefore may enhance, for example, the formation of a dynamic spindle matrix [7], which has been suggested to be important for a coordinated transition through mitosis.

Having established the existence of an anisotropic mitotic diffusion of macromolecules, it will be interesting to examine in subsequent work the influence of this phenomenon on a variety of specific mitotic signaling networks. Prime examples in this endeavor could be the several instances of the spindle assembly checkpoint and the associated wait-anaphase signal, which are both key for proper progress through mitosis. Moreover, our data indicate that simulation approaches to study mitosis need to include refined diffusional transport, i.e., anisotropic and even anomalous diffusion due to viscoelasticity.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.06.072.

Author Contributions

M.W. designed and conceived the project. N.P. and C.D. collected the data. N.P., C.D., and M.W. analyzed the data and wrote the manuscript.

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