Fluorescent particle tracking is a powerful technique for studying intracellular transport and micro rheological properties within living cells, which is most cases employs exogenous fluorescent tracer particles delivered into cells or fluorescent staining of cell organelles. Herein, we propose an alternative strategy, which is based on the generation of fluorescent species in situ with ultrashort laser pulses. Using mouse germinal vesicle oocytes as a model object, we demonstrate that femtosecond laser irradiation produces compact dense areas in the intracellular material containing fluorescent carbon dots synthesized from biological molecules. These dots have tunable persistent and excitation-dependent emission, which is highly advantageous for fluorescent imaging. We further show that tight focusing and tuning of irradiation parameters allow precise control of the location and size of fluorescently labeled areas and minimization of damage inflicted to cells. Pieces of the intracellular material down to the submicrometer size can be labeled with laser-produced fluorescent dots in real time and then employed as probes for detecting intracellular motion activity via fluorescent tracking. Analyzing their diffusion in the oocyte cytoplasm, we arrive to realistic characteristics of active forces generated within the cell and frequency-dependent shear modulus of the cytoplasm. We also quantitatively characterize the level of metabolic activity and density of the cytoskeleton meshwork. Our findings establish a new technique for probing intracellular mechanical properties and also promise applications in tracking individual cells in population or studies of spatiotemporal cell organization.
mostly propelled by ATP-dependent active processes within the cells rather than by thermal forces. A more advanced method called active microrheology measures a response of a tracer particle to a calibrated magnetic or optical force to retrieve local viscous and elastic moduli. Using the measured moduli, a frequency spectrum of forces acting upon an individual particle can be calculated from its measured displacement—a technique known as force spectrum microscopy.

Normally, intracellular particle tracking requires delivery of exogenous fluorescent particles into cells. Their large size prevents them from permeating cell membranes, whereas their internalization through endocytosis tends to lead to compartmentalization in endosomal vesicles or aggregation. Direct microinjection avoids endosomal entrapment but is tedious and physically damaging and can produce artifacts by affecting the cell’s state. Targeting a specific area of interest within the cell presents another challenge because intracellular migration of particles is difficult to control. Employment of fluorescently labeled organelles (i.e., mitochondria) proposed by some authors is still not free of limitations caused by their dynamic nature, localization in specific cell areas, presence of organelle-specific forces, and the labeling procedure itself.

An alternative technique, which can bypass many problems associated with the delivery of exogenous probes or labeling of endogenous organelles, is targeted production of fluorescent species (FS) in situ within cells. In this connection, tightly focused femtosecond laser pulses proved to be an especially efficient instrument for precise and localized modification of material properties, including the material of cells and tissues. A promising effect of femtosecond pulses irradiation was observed by several groups performing laser surgery, who found that it leaves brightly fluorescent areas in a variety of tissues including porcine cornea, embryos, mouse skeletal muscles, skin and brain, and collagen fibers. Only recently, Sun suggested the use of laser-generated FS as a labeling agent for a guided laser surgery of tissues in vivo. Here, we propose a different application of laser-mediated fluorescent labeling which focuses on live cell imaging and intracellular particle tracking. Taking advantage of the spatially localized character of nonlinear absorption, we strive to minimize both the laser-labeled volume and laser damage in order to create a microscale fluorescent area or “fluorescent probe” within a single living cell while preserving its viability. Similar to exogenous fluorescent particles, motion of this probe can be tracked using the fluorescent imaging technique to retrieve information on intracellular dynamics and mechanical properties of the intracellular material. Mammalian oocytes at the germinal vesicle (GV) stage were chosen as a convenient model object owing to their large size, distinct intracellular structures (nucleus and nucleolus), abundance of well-visible vesicles, and their well-studied micromechanics.

We evaluate laser-produced fluorescent probes as an instrument for tracking intracellular motion activity and characterize their formation process, chemical composition, and fluorescent properties. Raman and fluorescence characterization indicate that laser-generated FS belong to the family of carbon dots (CDs) which gained increasing attention as a bioimaging agent owing to their bright and tunable fluorescence, photostability, and excellent biocompatibility. A number of production techniques of CDs were proposed which can be divided into top-down fragmentation of carbon materials and bottom-up synthesis from a variety of organic precursors including biomolecules like carbohydrates, proteins and DNA, and even organic waste. Our findings provide first evidence that laser-mediated bottom-up synthesis of CDs from organic molecules is possible in situ in living cells which opens new perspectives for applications in fluorescent bioimaging.

Figure 1. Microscale fluorescent spots produced in the intracellular material upon femtosecond laser irradiation. Images of the oocyte (a) before, and (b) after exposure to a laser pulse train: combination of brightfield and fluorescent image, fluorescence is shown in green color. Laser peak intensity was 1.2 TW/cm², train length—100 ms. Scale bar—20 μm. (c) Intensity of two-photon excited fluorescence from the irradiated area during exposure to a train of pulses at 1.2 TW/cm². Insets show respective images of the oocyte material around the laser-irradiated spot, green color shows laser-induced fluorescence; scale bar—10 μm. (d) Probability of fluorescent spot formation as a function of irradiation parameters; 40X objective, n = 30 for each data point. (e) Average diameter of the fluorescent spot as a function of irradiation parameters; 40X objective, n = 30 for each data point. (f) A sub-micrometer fluorescent spot produced in the oocyte’s cytoplasm, combination of brightfield, and fluorescent images. Spot width (fwhm) is less than 200 nm; scale bar—1 μm.
RESULTS AND DISCUSSION

Production of Fluorescent Species in Living Oocytes upon Femtosecond Laser Irradiation. First, we examined the effect of FS production under laser irradiation. Normally, the oocyte material was only weakly fluorescent because of the presence of endogenous fluorophores; however, after laser irradiation, a brightly fluorescent micrometer-sized spot was formed at the laser focal point (Figure 1a,b). Fluorescence intensity in the spot was typically dozens of times higher than that in non-irradiated material, evidencing that large quantities of FS were formed under laser irradiation. Accumulation of FS was also monitored during the laser exposure using two-photon fluorescence excited by femtosecond pulses (Figure 1c). Whereas the initial fluorescence intensity was negligible, there was several-fold and nearly monotonous increase in intensity which reached the maximal value at the end of exposure. This effect indicates that accumulation of FS is gradual and continues during the entire period of laser exposure. Furthermore, the cytoplasm remained intact and showed no signs of ablation, thermally induced damage, or boiling during the exposure, which suggests a moderate temperature increase during FS generation.

Similar effects of FS production were observed in three distinct regions of the oocyte with different compositions of biomolecules, namely, cytoplasm, nucleus, and nucleolus. Likewise, we observed that fluorescent spots could be produced by femtosecond laser irradiation in several types of somatic cells: embryo lung cells and THP1 human monocytes (Figure S1). The formation of fluorescent spots had a threshold-like dependence on laser pulse parameters: it required sufficiently high peak laser intensities and irradiation doses (Figure 1d). The characteristic diameter of the fluorescent spot varied greatly in different points of the oocyte even at the same irradiation parameters probably because of the heterogeneity of intracellular material. Nevertheless, we found a tendency for spots to become larger with an increase in laser intensity and irradiation dose (Figure 1e). With intensity and dose close to the threshold, we produced fluorescent spot of sub-micrometer diameter, smaller than the laser beam focal spot size (Figure 1f). The minimal measured diameter (FWHM) was smaller than 200 nm or 1/4 of the laser wavelength. This diameter is nearly equal to the theoretical resolution limit of the 1.4NA oil immersion objective used to capture the image, which suggests that the spot could be actually even smaller, and its real dimensions were not resolved because of a limited spatial resolution.

All these results demonstrate benefits of femtosecond lasers for fluorescent labeling. Tightly focused ultrashort laser pulses feature strongly spatially localized interaction with matter. Normally, nonlinear absorption mostly occurs in a focal area as small as a laser wavelength. A combination of nonlinear and threshold effects can push the size of the laser-processed area even further into a subdiffraction region; and spatial resolution of tens of nanometers can be achieved by tuning laser fluence. Our findings demonstrate that the same principles apply to laser fluorescent labeling: only a small microscale volume of the intracellular material is labeled with laser-generated species.

The size of the labeled region can be tuned by irradiation parameters, and the minimal obtained size was well in the subwavelength region—less than 1/4 of the laser wavelength. As suggested by studies of femtosecond laser processing, this laser-labeled volume can potentially be made even smaller and can reach the scale of tens of nanometers, thus enabling routine labeling of single organelles. Another advantage of the laser technique is very precise and targeted labeling, as illustrated by an ability to create regular microscale patterns within a cell (Figure S2).

Raman and Fluorescent Spectroscopy Characterization of Laser-Generated Species. Insight into the chemical nature of laser-generated species was obtained using spatially resolved Raman spectroscopy in situ. Raman spectra of a laser-generated fluorescent spot in the cytoplasm exhibited typical bands associated with carbonaceous materials; specifically, strong D and G bands with maxima at 1350 and 1590 cm\(^{-1}\), respectively, and a smaller 2D or G’ band with a maximum at 2700–2750 cm\(^{-1}\) (Figure 2a, Table S1). These carbon-related bands were always detected within the fluorescent spot while not detectable at any cell area outside it. This demonstrates conclusively that production of FS is accompanied by carbonization of the intracellular organic material. Similar effects of accumulation of carbonized products in biological species upon ultrashort laser irradiation have been observed previously. They also explain emergence of near-infrared (NIR) absorption in the laser-irradiated cytoplasm which we previously found in the mouse embryo and also observed in the present experiments, because unlike biological materials, carbonaceous species produced by laser irradiation can exhibit an appreciable absorption in the NIR region. Analysis of the Raman spectra reveals additional characteristics of the carbonized species. Here, the G band is characteristic of sp\(^3\)-hybridized carbon, while the D or disorder Raman band corresponds to breathing vibrations of six-member aromatic rings activated by the disorder in the crystalline structure, in particular, edges of sp\(^2\) hybridized carbon crystallites, and is indicative of the presence of six-
member aromatic rings. High intensity of the D band reveals a large topological disorder in the carbonized material. The ratio between the intensity of G and D bands and their spectral position corresponds to the nanocrystalline graphite stage on the amorphization trajectory, which means that the carbonized material is composed of nanosized clusters of sp²-hybridized graphitic carbon. The same structure is typical for graphene or CDs which also exhibit strong hybridized graphitic carbon. The same structure is typical for

μnm. (c) Scheme of the control group as a function of irradiation parameters. Continuous excitation at 462 nm. (d) Comparison of normalized photobleaching kinetics of endogenous and laser-generated FS in the cytoplasm under continuous excitation at 462 nm. (e) Percentage of GV oocytes that maturated to the MI phase after laser irradiation of the cytoplasm relative to the control group as a function of irradiation parameters. n = 30 in each group. The dashed line shows the maturation percentage in the control group.

Previously, a number of mechanisms were proposed to explain fluorescent product generation in biological tissues upon pulsed laser irradiation, including destruction of mitochondria, formation of CDs through laser-mediated carbonization, and formation of fluorescent products of Maillard reaction through laser-induced heating. Our results indicate that formation of FS in the intracellular material correlates with laser-induced carbonization and formation of nanosized carbon. Furthermore, laser-generated FS exhibit excitation-dependent fluorescence spectra that violate a usual Vavilov–Kasha rule, which is typical for most fluorescent CDs. All these findings suggest that CDs synthesized through laser-induced carbonization can be fully or partly responsible for laser-induced fluorescence. This suggestion contradicts to Qin et al. who attributed femtosecond laser generation of FS in tissues to Maillard reaction between amino acids and hydrocarbons induced by strong heating and found that it was independent of laser-induced carbonization. It can be speculated that laser generation of FS might include several different mechanisms dependent on laser parameters and irradiation regime. Qin et al. concluded that laser-generated FS in tissues were mostly produced at temperatures close to 200 °C. Smaller laser-induced heating in our experimental conditions could result in a minor role of thermal–driven reactions and synthesis mostly carried out by non-thermal mechanisms. It is known that electric discharge plasma is an effective carbonization agent, which induces synthesis of fluorescent CDs from various organic precursors at low temperatures. The same plasma-driven synthesis can be realized in a microscale low-density plasma generated by nonlinear absorption of femtosecond laser pulses in the
biological material.40 This assumption is corroborated by a previously reported effect of CD synthesis from aromatic molecules irradiated by laser pulses, which is possibly mediated by laser-generated plasma.41,42 CDs can be readily synthesized from amino acids and proteins of DNA by non-laser routes, for example, using microwave or hydrothermal methods.43–45 Our preliminary results indicate that femtosecond laser irradiation of solution of amino acids or lipids produces CDs with luminescent properties similar to those synthesized within oocytes, which points to these biomolecules as possible precursors for bottom-up laser synthesis of CDs.

**Evaluation of Laser-Produced Fluorescent Species as Live-Cell Labeling Agents.** We addressed several problems important from a standpoint of application of laser-produced FS in live cell imaging: longevity of fluorescent spots, photostability of FS, and damage and cytotoxicity effect induced by laser irradiation.

First, we studied how long a fluorescent spot remains within the cell after irradiation. For this purpose, oocytes were irradiated with laser pulses and then cultivated in a CO2 incubator. Fluorescent spots, generated in the oocyte’s cytoplasm and nucleus, remained well visible after a 24-hour incubation period (Figure 3a). Some changes in their size after incubation could be a result of an asymmetrical shape and a change of orientation, whereas their fluorescence retained a large part of its original intensity (Figure S8). Hence, laser-generated FS mostly remained localized in a small laser-irradiated area and were not subjected to diffusive spreading. Our observations suggest that FS are embedded in a dense solid-like material, formed as a result of laser irradiation, which arrests their diffusion. The same conclusion is supported by measurements of time-dependent polarization emission anisotropy of FS, which was nearly constant throughout the interpulse period (12.5 ns, Figure S9), which means that rotational diffusion was also impeded and took time much larger than tens of nanoseconds. To further characterize physical changes in the laser-irradiated cytoplasm the oocytes, subjected to laser irradiation, were stained with a BODIPY fluorescent dye. We observed that dye molecules could penetrate and stain laser-modified areas. However, measurements of time-dependent emission anisotropy revealed a considerably slower molecular rotation in laser-irradiated regions, which indicates an increase in material viscosity (Figure 3b). The anisotropy decay kinetics was multi-exponential with at least two characteristic decay times. The short decay time is attributed to rotational re-orientation of unbound fluorophore molecules and the longer time — to slower re-orientation of fluorophore bound to biomolecules. Characteristic times of unbound BODIPY rotation were 0.54 ns in the normal cytoplasm compared with less than 300 ps reported for water solution46 and 1.6 ns in the laser-irradiated area, which indicates a nearly three-fold increase of effective viscosity. This increase was likely a result of laser-induced solidification discussed above. Inability of laser-produced FS to diffuse through laser-modified material suggests that they have much larger sizes than BODIPY molecules, which supports our conclusion that FS are fluorescent carbon nanoparticles.

It can be concluded that femtosecond laser irradiation produces two effects in the intracellular material: it locally labels it with FS, and it also causes local solidification so that a microscale solid-like fluorescent particle is formed in the irradiated volume of the intracellular material (Figure 3c). The size and shape of this particle are determined by laser irradiation parameters and geometrical characteristics of the focal area. We observed that small laser-produced fluorescent spots frequently had an ellipse-like shape, possibly reflecting the prolate ellipsoid shape of the focal area.

At the next stage we analyzed photostability of laser-synthesized FS. For comparative study of FS photobleaching an oocyte with a laser-generated fluorescent spot was subjected to continuous widefield irradiation at 462 nm, and time-dependent emission intensity was registered at the spot and a neighboring area of the cytoplasm. As shown in Figure 3d, the autofluorescence emission intensity decreased nearly 10-fold after several minutes of irradiation. By contrast, under the same conditions, fluorescence of laser-generated FS after an initial decay stabilized at a constant level equal to about 25–30% of the initial value, and considerable fluorescence signal was detected even after 20 min of continuous excitation. Thus, FS include a component strongly resistant to photobleaching, and in general, their photostability is superior to cell-endogenous fluorophores. The resistance to photobleaching probably results from high photostability of fluorescent CDs.47 The presence of photostable FS facilitates long-term fluorescent imaging and discrimination versus autofluorescence background.

Finally, we quantitatively evaluated the cytotoxic effect of femtosecond laser irradiation and its chemical products, including FS, using maturation to the MII phase as a simple criterion of oocyte viability. A large part of the oocytes subjected to laser irradiation and to formation of fluorescent spots were able to mature to the MII phase, as demonstrated by polar body extrusion (Figure 3a) and appearance of the metaphase plate in fluorescent images of Hoechst-stained oocytes (Figure S10). The maturation rate of the laser-irradiated oocytes was similar to the control group (Figure 3e), with the average maturation rate equal to 88% of the control level, and a p-value of the Fischer’s exact test for maturation in the irradiated and control groups equal to 0.2, thus supporting a lack of statistical effect of irradiation on the maturation rate at irradiation parameters indicated. Although exposure of oocytes to femtosecond laser pulses might produce local heating and leads to formation of plasma-mediated reaction products, including free radicals and reactive oxygen species,48 these negative effects do not dramatically affect oocytes developmental competence and do not interfere globally with oocyte viability. The same applies to toxicity of laser-synthesized FS. A limited damage inflicted by irradiation was probably a consequence of highly localized nonlinear light absorption discussed previously. Still, there is a question of a local effect of laser irradiation on integrity and functioning of organelles and elements of the cytoskeleton or nuclear matrix in and near the irradiated region which has to be addressed by further studies.

In summary, persistent fluorescence, structural stability, and limited cytotoxic effects make microscale fluorescent areas formed by femtosecond laser irradiation highly advantageous for fluorescent live-cell imaging.

**Particle Tracking and Analysis of Intracellular Mobility of Laser-Produced Fluorescent Probes.** Long-term stability of the laser-labeled area means that it can be effectively employed as a probe to track intracellular motion in a similar way to exogenous fluorescent probes. We analyzed dynamics of these artificial probes and information it provides on their environment. Microscale fluorescent probes produced in the oocyte cytoplasm by laser irradiation underwent a
Calculated complex shear modulus $G'(\omega)$ and viscous $G''$ components as a function of frequency calculated from averaged MSD($\tau$) $\times R_0$ for laser-generated probes in the ATP-depleted oocyte.

Figure 4. Intracellular fluorescence tracking of laser-produced probes and analysis of their mobility. (a) Sample diffusion track of a laser-generated fluorescent probe in the cytoplasm of a living oocyte recorded over 2 min, the scale bars are 20, 5, and 0.2 $\mu$m. (b) Sample native vesicles in the cytoplasm, the scale bar is 5 $\mu$m. (c) MSD as a function of lag time for individual tracks in the cytoplasm (dashed lines) and averaged function (solid line). (d) Difference in the long-time behavior of the MSD($\tau$) functions and diffusion tracks of two individual probes in the same oocyte. One of the probes undergoes a directed drift on a long time scale. (e) Averaged MSD multiplied by the particle radius as a function of lag time for motors. (f) Calculated complex shear modulus $G'$ and its elastic $G'$ and viscous $G''$ components as a function of frequency calculated from averaged MSD($\tau$) $\times R_0$ for laser-generated probes in the ATP-depleted oocyte.

Chaotic Brownian-like motion on a time scale from tenths of seconds to minutes which was well visible in fluorescent video recording (Figure 4a). We used a fluorescent tracking routine to retrieve trajectories of small probes with diameters ranging from 0.3 to 0.8 $\mu$m and calculated the mean square deviation (MSD) as a function of lag time to analyze their dynamics. The resulting MSD($\tau$) function can be fit with a power function $Ar^\alpha$. For all probes, a power parameter $\alpha$ on a timescale from 0.1 to 10 s was close to 0.5 (Figure 4c). At the same time, the pre-exponential factor $A$ demonstrated large variations by about an order of magnitude between different oocytes and even between different probes in the same area of the same oocyte. These variations were larger than the difference in diameters of the probes, which was limited by a factor of 2. It can be suggested that variation of the pre-exponential parameters reveals the heterogeneity of local mechanical properties. At lag times larger than approximately 10 s, MSD($\tau$) could switch to a growth faster than $r^2$, and for some trajectories, we observed a superdiffusive behavior with the power parameter $\alpha$ larger than 1 (Figure 4d). Trajectories of those superdiffusive particles were nonsymmetrical with an obvious drift direction, and that was an evidence of a motion driven by directed active processes, for example, slow remodeling of the cytoplasm meshwork resulting from polymerization of fibers or trafficking induced by molecular motors.

To check if the tracking of laser-generated fluorescent probes provides an adequate representation of dynamics of small objects embedded within the cytoplasm, we also analyzed motion of endogenous intracellular vesicles. These small objects with typical diameters from 0.5 to 1 $\mu$m were abundant in the cytoplasm and were easily detectable on brightfield microscopy images (Figure 4b) from which their coordinates can be retrieved using the same routine as employed for fluorescent probes. Recorded motion of all vesicles followed an MSD scaling characteristic of confined diffusion in agreement with subdiffusive motion of oocyte vesicles observed by Ahmed et al. The vesicles MSD scaled with lag time as $\tau^0$, which coincided with the scaling law we found for laser-generated probes. Because the mean square displacement scales inversely proportional to the particle radius, we averaged the MSD $\times R_0$ value over a series of measurements to take into account variations in the particle size, where the radius $R_0$ was determined for each individual particle from microscopy images. The averaged MSD($\tau$) $\times R_0$ for vesicles and laser-generated probes were generally similar, although the values for vesicles were larger by about 50% (Figure 4e). This discrepancy could arise from a systematic difference between hydrodynamic diameters of the probes and diameters measured from fluorescent images. Considering large variation between individual probes, this similarity of averaged deviations should be viewed as a good correspondence, and it demonstrates that motion of laser-generated probes and native cellular structures follows the same pattern.

To verify the nature of forces experienced by particles in the cytoplasm, we performed tracking of intracellular diffusion of both laser-generated probes and vesicles in oocytes kept at room temperature instead of physiological temperature (21 and 37 °C, respectively). We found that decrease in temperature had the most dramatic effect on mobility of particles as their averaged deviation became 6–7 times smaller. Interestingly, MSD $\times R_0$ values for artificial probes and vesicles, and their lag time dependences remained similar even at room temperature. A decrease of temperature by 16° must have only a small effect on purely thermal motion. Consequently, strong temperature dependence provides conclusive evidence that the diffusion of probes in the cytoplasm is mostly driven by active forces, generated in...
cells as a result biochemical reactions that have an Arrhenius dependence on temperature. In particular, the activity of myosin V, the motor which propels oscillations of the actin network in the mouse oocyte cytoplasm, decreases by a factor of 2.5 with a decrease of temperature from 37 °C to room temperature.\(^{10}\) Because MSD is proportional to a square of the force acting upon a particle, this decrease of forces generated by myosin motors must lead to a 6−7 fold decrease in MSD—in good accordance with our measurements.

A key question for the present work is how adequately laser-generated tracers can probe mechanical properties and forces generated in the intracellular material. Normally, mesoscale (tens of nanometers or larger) particles in cytoplasm cannot freely diffuse but are trapped in the cytoskeleton meshwork, and their motion occurs through deformation or contraction of fibers and reflects mechanical characteristic of the meshwork.\(^3\) The same meshwork plays another important function in oocytes as modulations of actin fibers, caused by myosin motors, creates constant agitation of the cytoskeleton which imparts stochastic active forces to objects embedded in the cytoplasm, and propels their random motion. Whereas this motion have an appearance of the Brownian diffusion, many recent works demonstrated that it is mostly caused by activity of molecular motors, in particular, myosin, and the active forces acting upon cytoplasm objects completely dominate thermal forces in the low-frequency range.\(^{8,9}\) The same is true in regard to laser-generated probes, as their motion was strongly dependent on temperature-dependent activity of molecular motors. The question remains whether there is some peculiarity in forces experienced by laser-generated probes. The laser irradiation can induce local damage to the cytoskeleton, for example, breaking and depolymerization of fibers as a result of thermal effects that can alter interaction of particles with the cytoskeleton. Also, laser-produced species can experience specific interactions with biomolecules or adhesion to intracellular components which could affect their dynamics. Tracer particles employed in fluorescent particle tracking are frequently covered with the passivating agent (e.g., PEG) to exclude these interactions.\(^{3,5,4}\) Naturally, with probes generated in situ control of surface chemistry is more problematic. Whereas a further study is needed to clarify these potential effects, our results suggest that, if present, they are of moderate scale and do not dramatically affect dynamics of laser-generated particles. It can be hypothesized that the same local character of nonlinear laser absorption, which determines miniature dimensions of the fluorescent probe, also limits damage inflicted to the cytoskeleton in the adjacent area so that its local architecture remains essentially unaltered. A good agreement between both magnitude and frequency dependence of MSD of native vesicles and laser-generated particles indicates that they sense essentially similar forces: both active forces from molecular motors activity and viscoelastic forces from the cytoplasm material. Hence, laser-generated tracer particles can be an instrument to probe intracellular forces and local mechanical properties of the intracellular material as adequate and precise as exogenous particles or endogenous vesicles or organelles routinely employed in cell microhology. Whereas large well-visible vesicles, which could be employed as natural tracers, are abundant in inner cytoplasm of the mouse oocyte, they are rare or completely absent in other regions of the cell, for example, cortical and perinuclear regions of the cytoplasm or nucleus and nucleolus. Similarly, other cell types can lack such natural probes. Laser generation of FS solves this problem by creating artificial probes in those cell types or cellular regions which do not have native tracer particles.

**Analysis of Intracellular Active Forces and Viscoelastic Properties of the Cytoplasm.** We further analyzed the MSD scaling and related it to the viscoelastic properties of the cytoplasm. Interestingly, proportionality of MSD of the particle in the cytoplasm to \(t^{0.5}\) was universal and independent of the type of particle and temperature-dependent magnitude of active forces. It seemingly reflects some fundamental characteristics of the cytoplasm material. This constant scaling can be understood by addressing the mechanical model of the oocyte cytoplasm developed by Ahmed et al.\(^{19}\) According to the model, uncorrelated activity of many myosin motors creates a constant remodeling of the cytoskeleton meshwork, which changes equilibrium positions of particles embedded in the meshwork, and imparts mechanical forces driving them to new equilibrium positions. At times larger than the duration of the force kick of molecular motors, these forces are determined solely by viscoelastic response of the cytoplasm material. Therefore, at small frequencies, the active forces spectrum scales with a power parameter dependent on the viscoelastic properties of the cytoplasm: \(f^2(\omega) \approx \nu^{-\beta}\), where parameter \(\beta\) characterizes frequency dependence of the complex shear modulus of the cytoplasm \(|G| \approx \nu^\beta\).

In the frequency range of our measurements, thermal forces can be neglected because both previous works and our study demonstrated that they are much smaller than forces generated by active processes. Time-dependent MSD of the particle can be related to the force spectrum and shear modulus using a modified Hooke’s law: \(x^2(\tau) \approx \nu^{-\beta}\)\(^4\) (Supporting Information Note 1).

Hence, from a \(t^{0.5}\) scaling found experimentally, the parameter \(\beta\) is equal to 3/8 \(\approx 0.38\). This parameter appears to be an intrinsic characteristic of the cytoplasm material, which is independent or weakly dependent on temperature or location within the cytoplasm, except specific zones in the cortex and close to the nuclear envelope, which will be discussed later, and it determines the constant scaling of the MSD function for all mesoscale objects of similar dimensions in the cytoplasm.

We observed that changes of metabolic activity in ATP-depleted oocytes that were incubated for extended period in substrate-free media\(^{35}\) could result in deviations of the measured MSD from a normal \(t^{0.5}\) scaling. Here, the power parameter was equal to about 0.35 at short times and only showed an upturn at a lag time longer than 10 s, indicating some residual directed motion of the of the same type as detected in normal oocytes (Figure 4e). Characteristically, the magnitude of MSD was also several times lesser than that in normal oocytes even at room temperature. We suggest that ATP depletion led to an almost complete halt in molecular motors activity which mostly eliminated active forces acting upon the probes. This reduction of forces propelling probes motion to purely thermal forces explains a large decrease in MSD amplitude. It can be derived from the fluctuation−dissipation theorem that when diffusion is driven by purely thermal forces, the MSD scaling is determined by the frequency dependence of the complex shear modulus: MSD \(\approx t^\beta\), where \(\beta\) is a power parameter in the complex modulus frequency dependence \(|G| \approx \nu^\beta\).\(^{53}\) Estimate of this parameter derived earlier (0.38) is in very good agreement with an experimental scaling of MSD (0.35). This agreement confirms...
validity of our assumption that in ATP-depleted oocytes thermal forces dominate over active forces produced by molecular motors, and it also suggests that viscoelastic properties of the cytoplasm were not strongly affected by elimination of active forces. When active forces acting upon the particles are negligible compared with thermal forces, frequency-dependent elastic $G'$ and viscous $G''$ moduli of the cytoplasm can be calculated from a measured MSD($τ$) following an established procedure. We applied this procedure to retrieve the complex shear modulus $G$ and its elastic and viscous parts $G'$ and $G''$ as functions of frequency in the range $0.1–5$ Hz (the lower limit was determined by an onset of directed motion), as shown on Figure 4f. These dependencies demonstrate that the cytoplasm material is relatively soft as indicated by small absolute values, that it is more elastic than viscous, but still the ratio between elastic and viscous moduli is not larger than 2 and that both moduli are monotonically increasing functions of the frequency. As explained above, this frequency dependence of the complex modulus was generally proportional to $ν^{0.35}$, coinciding with scaling of the MSD($τ$) function. Remarkably, the calculated values of $G'$ and $G''$ and the relation between them are in a reasonable agreement with those measured by Ahmed et al. using the optical tweezers technique which confirms the validity of our method.

Finally, we explored how measurements of mobility of fluorescent probes can reveal difference in cytoskeleton organization in specific areas of the cell. To this end, we have created a series of fluorescent probes in two regions which are known to feature especially dense network of actin filaments: the perinuclear region around the nucleus (GV) and the cell cortex on the inner face of the oocyte membrane (Figure 5a,b). In both regions, mobility of the probes was strongly restricted, and measured MSD magnitudes were much smaller than in inner cytoplasm between perinuclear region and the cortex (Figure 5c). Also, the MSD demonstrated weak dependence on the lag time. These results indicate that the dense fiber network in both regions gives them strongly elastic properties, and particles embedded in this dense network experience strong elastic trapping and cannot freely diffuse as opposed to a viscoelastic inner cytoplasm. This elastic trapping effect was apparently stronger in the cell cortex than in the perinuclear region, indicating a larger value of elastic modulus. Unlike the inner cytoplasm, we do not have a model to quantitatively characterize active forces generated in these specific areas, but still it can be concluded that our method can probe variations in rheological properties of the cell regions with a distinctly different cytoskeleton organization. This experiment demonstrates that the laser technique can precisely target specific regions of the cell (see also Figure S2), even those whose mechanical stiffness restricts access of tracer particles by diffusion. It also illustrates that laser irradiation can produce tracer particles in the cell regions where native tracers suitable for tracking are rare or absent.

### CONCLUSIONS

We have demonstrated a new approach to intracellular fluorescent particle tracking, which relies on FS locally produced by femtosecond laser pulses in the intracellular material. This technique provides an unprecedented capability to label in real time and with high accuracy a specific microscale volume as small as hundreds of nanometers within a living cell while preserving their viability. Fluorescence and Raman characterization reveals that laser-generated FS are CDs produced by carbonization of organic materials, and they exhibit bright and persistent excitation-dependent fluorescence. In addition to generation of FS, laser irradiation induces local densification of the intracellular material and formation of solid-like fluorescent particles. We demonstrate that these particles can be employed as probes for passive intracellular microrheology to retrieve information on mechanical properties and active forces generated in the intracellular material. Simple, fast, targeted, localized, and noninvasive fluorescent labeling can be adapted with great benefit to other tasks including labeling and tracking of individual cells in populations, cell lineage tracing, labeling and tracking of individual organelles within cells, studies of spatiotemporal organization during cell cycle, division, and fusion, active microrheology, and embryo development.

### METHODS

**Laser and Microscopy Setup.** Femtosecond laser pulses with a 80 MHz repetition rate and energy up to 25 nJ were generated using a Titanium–Sapphire oscillator (Tsunami, Spectra-Physics) pumped by a DPSS Nd:YVO$_4$ CW laser with 532 nm wavelength (Millennia Prime 63), Spectra-Physics). The central wavelength varied from 690 to 990 nm. The average laser power was tuned with a polarizing attenuator consisting of a half-wave plate and a polarizing cube. The maximum average power before the objective lens was 700 mW. The laser pulse trains were coupled to an inverted optical microscope (Olympus IX71) using a dielectric filter (Thorlabs FESH0750) mounted at 45° and then focused by a 40×
0.75NA (UPlanFLN, Olympus) or 100 × 1.4NA oil-immersion objective lens (UPlanSApo, Olympus) on a sample, which was placed on a three-axis stage. The laser beam completely filled the aperture of the objective. Taking the beam quality factor $M^2$ as 1, the focal spot diameter is estimated as $d = 1.22λ/NA ≈ 1.3 μm$ and confocal parameter as $b = πδ^2/2λ = 3.3 μm$ for the 40× objective lens and 0.68 and 0.93 μm, respectively, for the 100× objective lens. The pulse duration in the focal plane was measured by the Avesta AA-M autocorrelator and was equal to 25 fs. The SF11 prism compressor was used to compensate for the group velocity dispersion in the objective lens and other optical elements. The length of the pulse trains was determined by the opening time of a mechanical shutter (SH05, Thorlabs) unblocking the femtosecond laser beam for a time up to 200 ms. The image pixel size was 50 nm, and the typical frame duration in the focal plane was measured by the Avesta AA-M autocorrelator and was equal to 25 fs. The SF11 prism compressor was used to compensate for the group velocity dispersion in the objective lens and other optical elements. The length of the pulse trains was determined by the opening time of a mechanical shutter (SH05, Thorlabs) unblocking the femtosecond laser beam for a time up to 200 ms. The video-processing software. Briefly, the image was smoothed with a median filter, and then, a template was scanned across the image, and a coordinate corresponding to the best fit was found using an optimization procedure for each image. The discretization step was 1/16 of the image pixel size or approximately 3 nm. Localization routine precision was estimated by imaging of stationary 50 nm fluorescent beads lying on a glass substrate and was equal to approximately 4 nm. A time-averaged mean square deviation as a function of lag time $τ$ was calculated from the 2D tracks as $Δ2(τ) = (x(t + τ) − x(t))^2 + (y(t + τ) − y(t))^2$. The average was calculated over times $t$ from 0 to $T − τ$, where $T$ is the sequence length.

Trace particle radius was calculated from its size on fluorescence or brightfield image. For non-spherical particles, the shape was assumed to be a prolate ellipsoid, and the effective radius was calculated as $(R_x \times R_y)\frac{1}{2}$ where $R_1$ and $R_2$ are the length of semi-major and semi-minor axes, respectively.

Elastic $G'$ and viscous $G''$ moduli were calculated from MSD of particles subject to passive diffusion using relations $G'(ν) = 1G(ν)\sin(πa/2)$, $G''(ν) = 1G(ν)\sin(πa/2)$, where $G(ν) = 2k_BT/6nr_{R2}MSD(1/ν)Γ(1 + α(ν))$, $r_0$ is the effective radius of the particle, $Γ$ is the gamma function, and $α(ν)$ is the local logarithmic slope of the MSD($τ$) estimated at the frequency of interest.

**Raman Spectroscopy.** Raman spectra were collected in situ in living oocytes. Raman scattering was excited with a 532 nm DPSS CW laser (Coherent). Laser power at the focal spot was kept between 1 and 10 mW to avoid laser damage to the sample. The Raman signal was collected by the objective and registered by the Renishaw 1000B micro-Raman spectrometer attached to the microscope. Spectra were averaged over three different oocytes. Collected spectra were background-corrected and normalized at the intensity of the water OH stretching peak at 3400 cm$^{-1}$.

**Oocytes Collection and Cultivation.** All experiments with mice described in the present work were carried out under the supervision of the Institute of Chemical Physics RAS. Ethics Committee approved the experimental protocols.

CBA/C57Bl female hybrid mice aged 1–1.5 month were injected 10 IU pregnant mare serum gonadotropin (A036A02 “Intervet”) 48 h before oocyte collection. Injected females were killed by cervical dislocation. The ovaries were recovered from mice and placed into 2 mL of warm phosphate-buffered saline (PBS) buffer solution (D4031 “Sigma”) in the 35 mm Petri dishes (353001 “Falcon”). Cumulus-oocyte complexes (COCs) were extracted from ovaries and placed in M2 medium (M7167 “Sigma”) containing 0.1% hyaluronidase (H4272 “Sigma”) to remove cumulus cells. Then, oocytes were washed in PBS solution and moved into a 50 μL drop of PBS medium in a Petri dish with a glass and a center hole (100350 “SPL Lifesciences”), covered with 2.5–3 mL mineral oil (M8410 “Sigma”) and immediately placed on the microscope sample stage. For ATP-depletion experiments, oocytes were incubated for a long period (30 min or more) in a drop of PBS before measurements. During the laser treatment and tracking experiments, oocytes were either kept at physiological temperature (37°C) using a custom-made heating stage or were left at room temperature (21°C) when the heating stage was turned off.
After the laser treatment, oocytes were washed in M2 medium, transferred to four-well plastic dishes (30004 “SPL Lifesciences”) with 0.7 mL of IVM medium and cultivated in vitro in the CO2 incubator at 37 °C with 5% CO2. IVM culture medium composed of DMEM (C420 “PanEco”) supplemented with 15% fetal bovine serum (I31966-021 “Gibco”), 1.5 IU/mL gentamycin (G1272 “Sigma”), and 1 IU/mL pregnant mare serum gonadotropin (A036A02 “Intervet”). After overnight cultivation, oocytes were placed in M2 medium (M7167, Sigma) containing 5 μg/mL Hoechst 33342 stain (B2261, Sigma) for 10 min, then flushed in M2 medium, and examined for maturation to the metaphase II stage detected by the presence of a polar body and metaphase plate by fluorescence microscopy. Fluorescent images of oocytes were acquired using a Biomed 4 PR LUM microscope and Ximea xID MD061CU-SY camera.

For fluorescent staining, a solution of BODIPY 492/503 fluorophore in ethanol (1 mg/mL) was diluted by M2 medium in 0.2–0.5% volume ratio. Oocytes were incubated in diluted BODIPY solution at 37 °C for 15–25 min, washed with M2 medium and PBS buffer solution, and placed in PBS solution in a Petri dish.

The Fisher exact test for a 2 × 2 contingency table was used to identify the relationship between irradiation and oocyte maturation. For this purpose, the sum of the numbers of matured and non-matured oocytes in all irradiated groups was juxtaposed to the control group, in which oocytes were not subjected to laser irradiation but were kept under the same conditions. The number of oocytes was 260 in the irradiated group and 230 in the control group.

Frozen THP1 cells (human monocytic cells) were thawed in a 37 °C water bath and then diluted with pre-warmed medium (DMEM supplemented with 10% fetal bovine serum). Cell suspension was centrifuged at approximately 200G for 5 min. The supernatant was decanted, and the cell pellet was resuspended with M2 (Sigma, M7167) medium. During the experiments, cells were kept in M2 medium on the ice to avoid cell adhesion.

## ASSOCIATED CONTENT

1. Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01535.

Images of somatic cells with laser-produced fluorescent spots, tabulated parameters of Raman bands of laser-produced species, additional data for the characterization of FS fluorescent properties, changes in the area and emission intensity of fluorescent spots after incubation, illustrations of oocyte maturation to the MII phase and precise fluorescent patterning of the oocyte cytoplasm; and derivation of the MSD of an actively diffusing particle in the oocyte cytoplasm (PDF).

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### Author Contributions

V.A.N. conceived the experiment design. A.A.A. and A.M.S conducted laser irradiation experiments, and wrote the manuscript. A.A.A. conducted fluorescence measurements and analyzed tracking data. A.A.O., U.A.T. and M.S.S. collected and handled oocytes. A.O.O. collected and analyzed oocyte maturation statistics. M.S.S. processed intracellular tracking data. A.M.K. conducted Raman spectra measurements.

### Notes

The authors declare no competing financial interest.

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