Optical Tweezers as a Sensor for Intracellular Mechanical Properties

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Abstract

Endothelial cells that form the endothelium lining of all blood vessels are under constant shear stress from the blood flow. They have the capability of sensing differences in fluid shear stress (mechanosensing) and changing this mechanical message into a biochemical signal cascade (mechanotransduction), leading to different biological responses that could lead to cardiovascular diseases such as atherosclerosis. To understand how shear stress from blood flow is transmitted throughout endothelial cells, the magnitude and distribution of forces within each cell need to be studied. We propose a novel methodology by which we can explore cellular functions and study cytoskeleton dynamics of living cells at the subcellular level with minimal invasion. The optical tweezers technique can be used to measure the mechanical properties of the cytoskeleton in the vicinity of organelles and cellular structures. Using endocytosed silica beads as probes, we were able to determine the frequency dependent mechanical properties of the interior of cultured bovine endothelial cells. We treated the cytoskeleton with drugs that alter its composition to obtain the contribution of each cytoskeletal structure in the viscoelastic property of endothelial cells. Confocal images of drug treated cells are used as visual support for the optical tweezers results. In this talk, we will also present ongoing work on the application of the optical tweezers technique to examine the role of caveolae, which are invaginations of the plasma membrane, in mechanotransduction. Caveolae were labeled with three markers, caveolin-1, dynamin-2, and intersectin-1, in order to identify and target them as probes, and detect physical deformations in their vicinity, which are required for mechanotransduction.

Extended Abstract

Background

Mechanical stimuli throughout the blood circulation system are converted to biochemical signals that lead to various biological responses. This process, known as mechanotransduction, is carried out by endothelial cells that line all blood vessels. Depending on the local hemodynamics endothelial cells are exposed to, they undergo physical deformations at the cell surfaces, which trigger local intracellular biochemical cascades that are transmitted by the cytoskeleton, leading to transcriptional and structural changes. The mechanism used by endothelial cells to sense their biomechanical environment, or the pathways they use to transmit these messages are not very well understood.

Hemodynamics dictates morphologies and functions of endothelial cells. Previous studies show that shear-stress distribution in the blood circulation system is very heterogeneous, making it very difficult to study its effects. This heterogeneity is caused by many factors, among which is arterial geometry. Endothelial cells experience different fluid shear-stresses from the blood flow, depending on their location in the circulation system. If we consider the case of atherosclerosis, there is a preferential plaque formation in regions of low shear stress (less than 5 dynes/cm^2) corresponding to regions of arterial curvature, where the flow pattern is no longer laminar. By the same token, regions exposed to laminar flow and high shear stress (greater than 15dynes/cm^2) are protected from this plaque formation (*Cunningham, K. et al.*)[1].

To study how endothelial cells are able to sense differences in shear stress, we turn to an *in vitro* system, were these cells are cultured and exposed to a uniform flow at physiological rates. When exposed to flow, endothelial cells undergo changes in their shape from cuboidal (no flow condition) to spindle-shaped, and rearrange their cytoskeleton to align their microfilaments in the direction of flow[2]. *Barakat, A. and Davies, F.*[3] used atomic force microscopy (AFM) to obtain shear stress distribution over these cells exposed to the same shear stress conditions. Their results show heterogeneity in shear stress distribution not only between cells that are next to each other, but also between short distances within the same cell. Therefore, different regions of the same cell experience blood flow differently. The distribution of shear forces throughout the cell is achieved by the cytoskeleton.

The cytoskeleton is a living polymer network that is distributed throughout the cytoplasm of the cell, and it plays a role in maintaining the cell's mechanical integrity. It is composed of three major types of filaments: actin filaments (or microfilaments), microtubules, and intermediate filaments. This three-dimensional network of proteins acts as both the muscle and the skeleton of cells. The cytoskeletal system is dynamic; it adapts to changes in the environment by constantly polymerizing and depolymerizing its components, depending on the necessary functions of the cell. The molecular and mechanical basis for the transmission of mechano-chemical stimuli through this network of cytoskeletal proteins is not well understood.[4] Quantifying the significance of the transmission of mechanical forces in the cytoskeleton by determining the micromechanical properties can facilitate a better understanding of the complex system.

Endothelial cells behave like soft materials, exhibiting both liquid-like and solid-like behaviors[5] when exposed to shear forces. When subjected to an oscillatory shear stress, these cells can store and dissipate mechanical energy. The amount of energy stored per unit volume by a material is a measure of its solid-like behavior; it is represented in terms of what is known as the *storage modulus* $G'(\omega)$ where ω is the oscillation frequency. Similarly, the energy dissipated per unit volume by a material is a measure of its liquid-like behavior; it is represented by the *loss modulus* $G''(\omega)$. The storage and loss moduli describe the mechanical properties of the materials. But unlike most soft materials (polymer gels), the interior of endothelial cells is inhomogeneous. Hence, conventional macro-rheology studies would not be sufficient to give information on the mechanical properties of such inhomogeneous systems. To study the microscopic mechanical properties inside the cell, micro-rheology studies are used. This methodology employs either thermal motions of tracer particles,[6] or the forced oscillation of micron-sized probe particles[7] residing inside the cell, to determine the mechanical properties of soft materials, in this case the cytoskeleton, surrounding the probe particles. We use oscillating optical tweezers based micro-rheology to study the mechanical properties of endothelial cell cytoskeleton.

Theoretical Background of Optical Tweezers

By strongly focusing a laser beam to create a highly localized electric field at the focal point, a large electric field gradient is formed in both the axial (the laser propagation direction) and radial directions around the laser focal point. A sufficiently steep field gradient can create a force large enough to counter Brownian motion of the particle, thus yielding a stable optical trap in all three dimensions. The balance of gradient and scattering forces in the axial direction causes the potential minimum for the trapped particle to be slightly downstream from the focal point of the lens. The achievement of axial stability, due to the availability of high numerical aperture (NA) objective lenses, makes the trap suitable for a wide range of applications. The magnitudes of the forces are generally quoted to be about 1 pico-Newton per milli-Watt (pN/mW) of laser power at the trap site. Because of their relatively noninvasive nature, laser tweezers are ideal for probing individual colloids and cells in their microscopic environments.

The radial trapping force can be obtained from the effective potential of the trapped particle in the Gaussian intensity profile of the laser beam

$$U = \frac{-3V_2 n_1}{c}\left(\frac{n_2^2 - n_1^2}{n_2^2 + 2n_1^2}\right) I_o\, e^{-r^2/R^2} \qquad (1)$$

where

- V_2: the volume of the particle
- c: the speed of light
- n_1: the index of refraction of the solvent
- n_2: the index of refraction of the particle
- I_o: the laser intensity
- R: the distance from the center of the laser beam where the light intensity is 1/e of the value at the center of the focal point.

The force F on a particle at a distance *r* from the center of the trap:

$$\vec{F} = -\Delta U = -\frac{6\, r\, V_2\, n_1}{c\, R^2} I_o \left(\frac{n_2^2 - n_1^2}{n_2^2 + 2\, n_1^2}\right) e^{-r^2/R^2}\, \hat{r} \equiv -k_{ot}\, r\, e^{-r^2/R^2}\, \hat{r} \qquad (2)$$

For small displacements, the force follows Hooke's law with a spring constant k_{ot}, *i.e.*, F= k_{ot} r. For a polystyrene sphere (n_2 = 1.57, radius a = 0.5 μm) in water (n_1=1.33) and the laser power at the trap (R = 0.5 μm) of 1 mW the spring constant is approximately 8 μN/m. At a displacement of r ~ 0.25R the force is approximately 1 pN.

The equation of motion for a particle trapped by an oscillating trap in a viscous medium is determined by the viscous drag force experienced by the particle and the force generated by the optical trap.[8] For a simple viscous liquid, the drag force is taken to be the Stokes drag, $F_{drag} = -6\pi\eta_0 a v$, where η_0 is the zero shear viscosity of the liquid, and *v* is the velocity of the particle. Newton's second law of motion, neglecting the inertia of the particle, leads to the equation of motion for the particle as

$$\sum F = -6\pi\eta_0 a\dot{x}(\omega,t) + k_{ot}[Ae^{i\omega t} - x(\omega,t)] = \xi(t) \qquad (3)$$

where ξ(t) is the Brownian force on the particle due to the surrounding medium. The above equation describes the motion of a particle of radius a as a damped, driven harmonic oscillator in thermal equilibrium. The steady-state solution of the above equation provides the time-dependent position of the particle, expressed as a function of oscillation frequency, ω, as

$$x(t,\omega) = x(\omega)e^{i\omega t} = D(\omega)e^{i(\omega t - \delta(\omega))} + X(\omega,t) \qquad (4)$$

where the amplitude of the displacement of the particle due to the external forcing is given by

$$D(\omega) = \frac{A}{\sqrt{1+\tau^2\omega^2}} \qquad (5)$$

with $\tau = 6\pi\eta_0 a / k_{ot}$ and the phase shift is given by

$$\delta(\omega) = \tan^{-1}(\tau\omega) \qquad (6)$$

and the auto-correlation function of the Brownian fluctuation of the particle *X* (t) is

$$\langle X(t)X(0)\rangle = \frac{k_B T}{k_{ot}} e^{-t/\tau}$$

with the power spectral density, given by[9]

$$\langle X(\omega)X(0)\rangle = \frac{2k_B T}{k_{ot}} \frac{\tau}{1+\tau^2\omega^2}$$

where $\langle X(\omega)X(0)\rangle$ is the time Fourier transform of $\langle X(t)X(0)\rangle$, k_B is Boltzmann constant, *T* is the absolute temperature, and $\tau = 6\pi\eta_0 a / k_{ot}$. Although the forced oscillation D can be smaller than the thermal fluctuation *X*, it is possible to separate the two by the lock-in detection method (see below).

Extension to the Applications when the Trapped Particle is in a Viscoelastic Medium:

To relate the motion of a single colloidal particle to the mechanical properties of the material surrounding the particle, we model the material as a viscoelastic substance with a frequency dependent viscosity and elasticity. Newton's second law of motion then leads to the equation of motion a particle in a viscoelastic fluid as:

$$\sum F = -6\pi a\eta(\omega)_{material}\frac{dx}{dt} - (k(\omega)_{material} + k_{ot})(Ae^{i\omega t} - x) = \xi(t) \qquad (7)$$

The storage and loss moduli $G'(\omega)$ and $G''(\omega)$ of the medium can be calculated from the phase shift and amplitude of the particle responding to the oscillating traps using the following relationships[10]

$$G'(\omega) = \frac{Ak_{ot}}{6\pi a}\left(\frac{\cos\delta(\omega)}{D(\omega)} - 1\right) \qquad (8)$$

$$G''(\omega) = \frac{Ak_{ot}}{6\pi a}\left(\frac{\sin\delta(\omega)}{D(\omega)}\right) \qquad (9)$$

The calibration of the effective spring constant of the trap is realized by fitting Eqs. (5) and (6) to the measured amplitude and phase of the displacement of a particle in water. Because the viscosity of water is known, the single fitting parameter k_{ot} gives the effective spring constant of the optical trap.

Determination of the absolute values of the measured G' or G'' depends on an accurate determination of the stiffness of the optical tweezers k_{ot}. For studies of biological cells, unless the exact nature of the organelles is known, k_{ot} cannot be determined independently. Nevertheless, we can calculate the k_{ot} value based on the assumption that the optical refractive index of the organelle structure is n = 1.45 (assuming 70% protein or lipids, and 30% water) and that of the surrounding cytoplasmic fluids (20% protein and 80% of water) is n = 1.37. The k_{ot} estimated is approximately 33% weaker than that of a comparable silica bead in water. It is important to note that the uncertainly in the refractive index of the trapped object can cause an error only in the pre-factor of the viscoelastic moduli and not the overall frequency or the time dependence of the spectra. Any organelle structural reorganization involving changes in protein/lipid content could change the optical trapping strength, and thus the apparent k_{ot}.

Optical Tweezers Setup

The experimental apparatus used to measure the motion of the trapped particle is shown in Figure 1; a more detailed description of the setup can be found elsewhere.[18,11] The optical tweezers are powered by a frequency-doubled, Nd:VO_4 diode-pumped laser at the wavelength of 532 nm. A telescope composed of two lenses, *F1* and *F2*, is used to expand the laser beam to the size of the back aperture of the microscope objective. A half wave plate, *HW*, rotates the direction of polarization of the linearly polarized laser beam to allow the control of the relative intensity of the two beams exiting the polarizing beam splitter, *PBS1*. The first beam, of *P* polarization, is reflected off a stationary mirror *M2*, and is used to create a stationary trap. The second beam, of *S* polarization, is steered by a high frequency oscillating mirror, *M1*, and forms the oscillating trap. A sinusoidal electric signal, generated by a

frequency synthesizer built-in to the lock-in amplifier, is fed into a piezoelectric driver to drive the steering mirror, *M1*. The two laser beams are recombined at the second polarizing beam splitter, *PBS2*, before entering the microscope. The optical traps are formed at the microscope sample chamber by focusing laser beams through a high numerical aperture microscope objective in an inverted microscope. Adjusting the mirrors *M1* and *M2* varies the separation distance between the two traps. The telescope, composed of the lens pair f_1-f_2, is arranged such that the locations of M_1 and M_2 are conjugate to the back aperture of the objective. This arrangement ensures that a pure rotation of the laser beam pivoted at M_1 or M_2 creates a pure rotation pivoted at the back aperture of the objective lens, thus ensuring that the laser intensity at the trap remains almost constant during oscillation.

The oscillating optical traps technique takes advantage of the use of a lock-in amplifier to determine the motion of a particle by measuring both the magnitude and phase of the displacement of a particle. To detect the motion of the trapped particles relative to the motion of the trap, the light diffracted from the trapped particles can be measured by a split photodiode.[12] In this case, the difference of photoelectric current from the two photodiodes is proportional to the displacement of the trapped particle. In the oscillating tweezers approach, the photoelectric current difference is fed into the lock-in amplifier where the signal that drives the oscillating mirror serves as the reference signal for the lock-in amplifier. With this arrangement, the lock-in amplifier measures both the magnitude of the displacement and the phase shift between the particle's displacement and the oscillating trap.

The split photodiode, at a location conjugate to the focal plane of the objective, is used to detect the light diffracted from the probing laser beam by the particle in the optical trap. The position of the particle, described by Eq. 4, contains signal from both the forced oscillation and the Brownian motion. The lock-in amplifier (Stanford Research SR-830) is used to selectively detect the forced oscillation. Basically, the SR-830 lock-in amplifier measures the amplitude and the phase of a sinusoidal voltage. The lock-in amplifier uses a reference signal (the voltage driving the oscillating mirror) synchronized with the signal to "find" the signal to be measured, while ignoring anything that is not synchronized with the reference. Although the Brownian motion of a particle has a Fourier component at the same frequency of the signal, since the Fourier component does not have a well defined phase, it will be rejected by the lock-in amplifier after signal averaging over a proper integration time. By this process, the lock-in can measure voltage amplitudes as small as a few nano volts, while ignoring non-synchronized noise signals even thousands of times larger. The phase shift measured by the lock-in amplifier is that of the signal of the forced oscillation.

Optical tweezers with a design shown in Fig. 1 can measure the dynamic mechanical modulus in the frequency range of 0.1-6,000 Hz. For time dependent measurements, the data can be taken at a rate as fast as 100 data points per second. Using the lock-in detection technique, we are able to measure the forced particle displacements on the order of 1 nanometer, much smaller than the magnitude of the Brownian motion of the particle at room temperature.

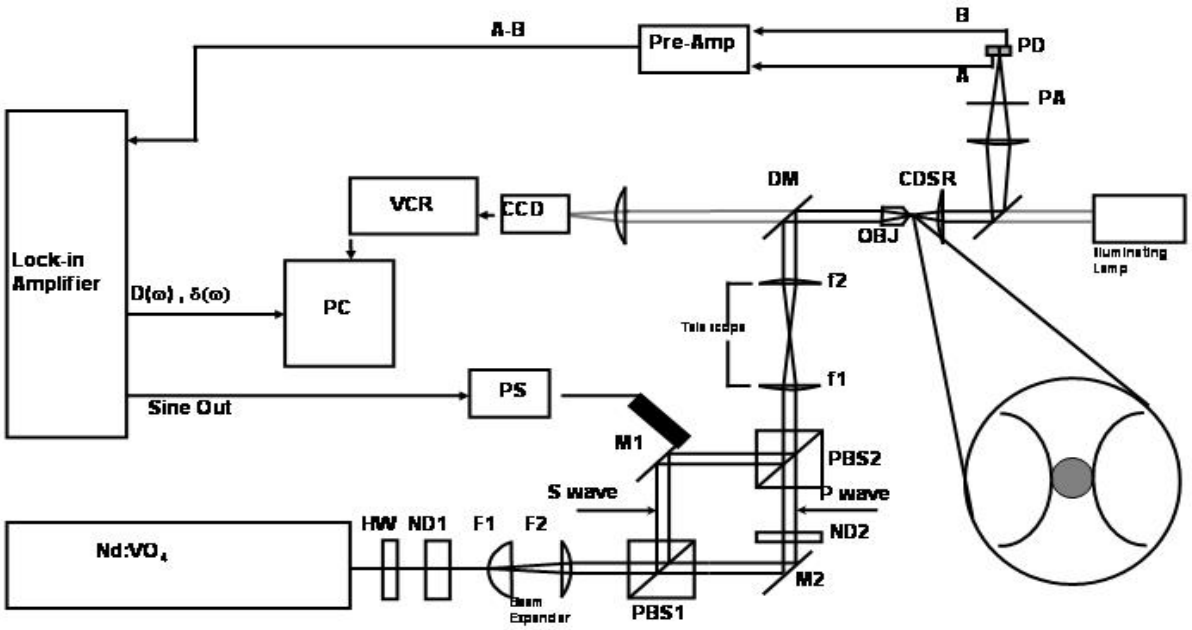

Figure 1. Schematic of the oscillating -tweezers setup. HW is a half-wave plate; ND is a neutral density filter; PBS is a polarizing beam splitter to separate the incoming linearly polarized laser beam into an S wave and a P wave beams; M1 is a piezoelectric-controlled mirror; PS is a piezoelectric power supply; the piezo-controlled mirror M1 produces the oscillating laser beam for creating the oscillating tweezer; the mirror M2 is stationary for producing the probe beam. DB is a dichroic mirror for allowing the laser beam to reflect and long wavelength illuminating light to pass; PA is a polarization analyzer used for blocking the oscillating laser beam from entering PD, the split photodiode. In the diagram, the sample is located immediately to the right of the objective lens OBJ.

Mechanical Properties of Bovine Artery Endothelial Cell Cytoskeleton

To obtain the intracellular visocoelastic properties of bovine vascular endothelial cells, we carried out several experiments. In the first experiment, we introduced silica beads by endocytosis to a culture of endothelial cells grown to 50-70% confluency. The silica beads were used as probes for optical traps, and their motion was tracked to obtain displacement and phase shift data. The output data will be computed to give the frequency dependent behavior of the cytoskeleton in the vicinity of the probe bead.

In a second set of experiments, we examine the contribution of the different cytoskeletal proteins in the overall behavior studied in the first experiment. To achieve this, we treated cultures of endothelial cells (after introduction of beads) with drugs that alter the composition of the cytoskeleton. One set of cells were treated with cytochalasin B, organic molecules and fungal toxins that cap the plus ends of actin filaments and cause them to depolymerize. Then, we measure the viscoelasticity of endothelial cells in the absence of microfilaments. A second drug treatment we use is nocodazole, an anti-microtubular drug that has the ability to depolymerize microtubules. Here, we measure the viscoelastic properties of the cells in the vicinity of beads in the absence of microtubules.

For a complete picture of intracellular dynamics, we couple our optical tweezers experiment with confocal microscopy studies. Both the drug treated and control cells were labeled with anti-actin and anti-tubulin antibodies to visualize the cytoskeletal changes the cell undergoes under these different conditions.

Future studies in this area will include the effect of flow on the viscoelastic properties of endothelial cells, using beads as probes for optical traps. We will also examine the effect of the drugs on the viscoelasticity of cells exposed to flow.

Examining the Role of Caveolae in Mechanotransduction

Although it is known that endothelial cells can sense their micro-mechanical environment and transmit the message throughout the cell, the mechanisms by which they carry out this function needs further exploring. One mechanism by which these cells are believed to sense fluid shear stress is through caveolae, which are invaginations of the cell membrane, that are responsible for transcytosis, the transport of macromolecules from the blood flow to the underlying tissue and vice versa.

Previous studies established caveolae as mechanosensors for the reasons that (i) they are responsive to changes in shear stress[13], (ii) they have linkage to the actin cytoskeleton, (iii) they have the capability to compartmentalize signaling molecules, and (iv) they are found to be associated with previously identified mechanosensors, such as G proteins, and ion channels, as well as signaling molecules such as eNOS and calcium (calcium ATPases and IP3 receptors)[14]. Using caveolae as probes for optical tweezers studies, we can measure the deformations the cytoskeleton undergoes in their vicinity.

Optical tweezers technique is a non-invasive methodology, which can be used to study viscoelastic properties of cells without the introduction of foreign particles such as beads. Using wide-field microscopy, granular structures which present index of refraction mismatch can be used as probes for viscoelastic measurements. To see if caveolae appear as distinct granular structures in the wide-field image of endothelial cells, we labeled 3 caveolar markers, caveolin-1, dynamin-2, and intersectin-1, with fluorescent tagged antibodies, and super-imposed these confocal fluorescent images with their corresponding wide-field images. Our results show that certain granular structures co-localize with these markers which indicate the presence of caveolae in the vicinity. These results established caveolae as potential probes for optical tweezers studies.

To study the mechanics of the cytoskeleton in the vicinity of caveolae, we need to be able to identify these structures, and simultaneously trap them with optical tweezers. We are presently developing an optical tweezers-based confocal imaging cytorheometer, which will allow us to achieve both. Since antibody labeling cannot be used in live cell studies, we reverted to GFP-labeling of these three caveolar markers, whose fluorescence signal will use to identify caveolae in the vicinity of granular structures. Once caveolar structures are located, the wide-field image can be used to guide the trapping of these structures, and further extraction of G' and G" values in its vicinity as a function of time and frequency can be obtained. The cytoskeletal change with time can also be visualized by GFP-labeling actin filaments and tracking their displacement using confocal microscopy.

Summary

In the first part of our work, we map out the viscoelastic properties of the cytoskeleton at different regions of the cell using silica beads as probes for optical tweezers measurements. We examine the contribution of microfilaments and microtubules in this viscoelastic property, by treating endothelial cells with drugs that alter the composition of these cytoskeletal proteins. The gathering of this crucial information will allow us to have a better understanding of the effect of fluid shear stress on endothelial cells, and their distribution throughout these cells.

The second part of our work deals with applying optical tweezers technique to study the mechanotransduction role of caveolae. Using the optical tweezers-based confocal imaging cytorheometer setup, we will be able to locate fluorescently labeled caveolar markers in the vicinity of granular structures with index of refraction mismatch, making them potential probes for optical tweezers studies. From this study, not only can we measure the change in viscoelastic properties around caveolae, but also visualize the physical deformations by labeling actin filaments and studying their change with time using confocal microscopy.

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