Development of a Near-infrared Laser-induced Surface Deformation Microscope and Its Application to the Dynamic Viscoelastic Measurements of Single Living Plant Cell Surfaces

Toshinori Morisaki,*1 Hitomi Onuki,*2 Kenji Hashimoto,*3 Kazuyuki Kuchitsu,*3,*4 and Hiroharu Yui*1,*2†

*1 Water Frontier Science & Technology Research Center, Research Institute for Science & Technology, Tokyo University of Science, 1-3 Kagurazaka, Shinjuku, Tokyo 162–8601, Japan
*2 Department of Chemistry, Faculty of Science, Tokyo University of Science, 1-3 Kagurazaka, Shinjuku, Tokyo 162–8601, Japan
*3 Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda 278–8510, Japan
*4 Imaging Frontier Center, Research Institute for Science & Technology, Tokyo University of Science, 2641 Yamazaki, Noda 278–8510, Japan

A near-infrared laser-induced surface deformation (NIR-LISD) microscope is developed and is applied to the dynamic viscoelastic measurements of the surface of a living plant cell. In the microscope, the deformation of the surface is induced by an NIR laser beam, and then the change in intensity of the probe beam reflected from the surface reflects its viscoelasticity. The application of the NIR laser beam has a great advantage for the prevention of damage to the plant cell compared to the irradiation of a visible laser beam in LISD measurements. The NIR-LISD microscope allows for discriminating the differences in power spectra between the subapical and lateral regions of single rhizoids. It is a useful method for the dynamic viscoelastic measurements of cells, such as plant cells, that are damaged due to the strong absorption of ultraviolet or visible light.

Keywords LISD microscope, near-infrared laser, dynamic viscoelasticity, plant cell

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Introduction

Viscoelasticity of cell surfaces in animals and plants closely contributes to the invasion of cancerous cells and to the structural integrity of plant tissues.1,2 For animal cells, their surfaces take a hierarchical structure composed of lipid bilayers, cortical actins developed two-dimensionally below the bilayers, and cytoskeletons conjugated with the bilayers.3 The surfaces in plant cells are covered with thick cell walls that mainly consist of cross-linked cellulose fibrils and pectin filling the gaps between the fibrils.4 Recently, it has been suggested that cell walls have not only an elastic nature but also a fluidic one, as observed from the flow behavior of single plant cells.5 Thus, for the elucidation of the complicated mechanical behavior of the cell surfaces in animals and plants that show both a fluidic and an elastic nature, the detection of the dynamic relaxation responses of the surfaces to the frequency of external forces is crucial.

Atomic force microscope (AFM) has been widely utilized for the detection of the dynamic relaxation responses of plasma membrane in animal cells.6–8 On the other hand, the mechanical behavior of cell walls has been limited to the detection of their static responses with AFM and the creep method.9,10 Although AFM is a powerful method for the dynamic viscoelastic measurements of cell surfaces, the upper frequency of applied external forces is limited to about 103 Hz.6–8 Dynamic viscoelastic measurements in the range above 103 Hz are required for the understanding of more locally varying viscoelasticity of cell surfaces.

We have developed a laser-induced surface deformation (LISD) microscope and have applied it to dynamic viscoelastic measurements of single living cells and of surfaces in micrometer-sized cellular structures.11–13 In the LISD, a laser beam is focused onto a surface, followed by the generation of radiation pressure. The radiation pressure induces deformation of the surface.14 With the developed microscope, we achieved spatial resolution of 1–2 μm in the horizontal direction, allowing the measurements in the sub-cellular-sized regions of cellular surfaces. Furthermore, by the non-contact forced oscillation of a surface with radiation pressure, the frequency range in dynamic viscoelastic measurements was extended up to 106 Hz.

However, in the LISD microscope, the cell viability was about 60% in the case of exposure of a pump beam of 60 mW for 20 min. to fibroblast cells.11 An increase in cell viability is required for diagnosis of cancerous cells and for understanding...
of the mechanism of tissue formation based on the rheological properties of cells. It is considered that the cell damage in the LISD measurements is caused by the optical absorption of biomolecules, such as lipids and hemeproteins, and water in a cell to an intense visible laser used for the inducement of the deformation of plasma membrane. Since pigments for photosynthesis such as chlorophyll contained in plant cells strongly absorb visible light, the exposure of an intense visible laser in LISD measurements also damages plant cells. To decrease cell damage, a light beam with wavelength that has relatively weak absorption to plant cell molecules and pigments should be applied for the deformation of cell surfaces.

Here we newly developed an LISD microscope with the use of a near-infrared (NIR) laser beam as pump beam (NIR-LISD microscope). In the NIR region, especially, in the region of wavelength from 700 to 900 nm, although optical absorption from water is larger than that in the visible region, the absorption from lipids, hemeproteins, and pigments for photosynthesis in plants is smaller. Thus, it is expected that the application of an NIR laser beam in LISD measurements will enable us to decrease cell damage. In this paper, the NIR-LISD microscope was applied to the dynamic viscoelastic measurements of surfaces in single plant cells. The tolerance of plant samples to the exposure of the NIR laser beam was also examined.

Experimental

Experimental setup of the NIR-LISD microscope

The principle of the LISD measurements has been already described in detail. Figure 1 shows the optical system of the NIR-LISD microscope. The frequency of intensity of a Ti:Sapphire laser (800 nm, 3.5 W, Coherent, MBR-110) (pump beam), pumped by a Nd:YVO₄ laser (532 nm, 18 W, Coherent, Verdi-V18), was modulated by an AOM (Model 3080-125, Crystal Technology Inc.) controlled with function synthesizer (Model 1915, NF Electronic Instruments). The pump beam was introduced to the objective lens at 70 mW. An objective (20×, N.A. = 0.45) was used for the focusing of the pump and probe beams. The pump beam was coaxially arranged with a He-Ne laser (632.8 nm, 0.5 mW, MELLES GRIOT, 25LHP213) (probe beam). The two beams were focused onto the sample surfaces using an objective (20×, N.A. = 0.45, OLYMPUS, LCPlan-N-IR or 60×, N.A. = 0.70, OLYMPUS, LUCPlanFL-N). They were positioned on a sample surface with an XY stage (BIOS-205T, SIGMA KOKI) in the horizontal direction and with an objective (100×, N.A. = 0.95, OLYMPUS, PlanFL) for depth direction. The intensity of the reflected probe beam (signal beam) from the surface is amplified by heterodyne interference with a split probe beam (local beam). The signal and local beams were detected by a photodiode (PD) (Model C10508, HAMAMATSU), then were processed with a fast Fourier transform (FFT) spectrum analyzer (Model 3056, Sony Tektronix). The LISD measurements were performed at 25°C. For NIR-LISD measurements, the wavelength of the pump beam was set to 800 nm. The diameter of the beam in the focal region was estimated from a CCD image to be about 4 or 1 μm with the use of the objective lens (20×, N.A. = 0.45 or 60×, N.A. = 0.70), respectively.

Sample preparation for the laser tolerance and NIR-LISD measurements

To examine the tolerance of plant tissues to the exposure of the pump beam, a green-colored rice leaf was placed on a glass-bottomed dish (Matsunami Glass Ind.). The pump beam with 532 or 800 nm of wavelength was focused onto the leaf surface. For the NIR-LISD measurements, gemmae were collected from wild-type *marchantia polymorpha*. The gemmae were cultured with a poly-L-lysine-coated glass-bottomed dish (Matsunami Glass Ind.) in 2 mL of culture medium under visible light irradiation. Rhizoids started growing from the gemmae after twenty hours of culture. After twenty-four hours of culture, the medium was changed with 200 μL of fresh culture medium. The medium surface was covered with a cover glass to prevent light scattering from the air/medium surface.

Results and Discussion

We verified the principle of LISD upon the application of an NIR laser beam as a pump beam. In principle, because surfaces of samples are periodically forced to oscillate with the
modulation frequency of the pump beam intensity, the reflected probe beam intensity from the surfaces is also modulated with the same frequency. Thus, when the pump and probe beams are irradiated onto the surfaces simultaneously, LISD signals are observed as the same frequency components by heterodyne interference. As a test sample for the verification of the LISD principle, \( n \)-tetradecane was measured. \( N \)-Tetradecane is a typical liquid with a fluidic surface. In the application of the LISD method to the surface, surface tension acts as a restoring force and viscosity as a friction force. Then, at relatively lower frequencies, surface deformation and relaxation are able to follow the frequencies of pump beam intensity, while at higher frequencies, large deformations are not able to follow, resulting in the slope of the power spectrum. Figures 2a and 2b show the frequency responses of the \( n \)-tetradecane surface to the irradiation by either the probe beam or the pump beam. In the simultaneous irradiation of the two beams onto the surface, the signals were observed in the frequencies (160, 200, and 240 kHz) where the pump beam intensity was modulated (Figs. 2c – 2e), indicating the detection of the LISD signals from the surface.

In the application of a periodical external force to a sample, its deformation follows the force at relatively lower frequencies, leading to the emergence of plateau displacement. At relatively higher frequencies, the deformation does not follow the force due to friction or viscous conditions, resulting in the decrease in displacement of a sample. The power spectrum of \( n \)-tetradecane was measured in the range from \( 10^3 \) to \( 10^6 \) Hz with the NIR-LISD microscope (Fig. 3, \( \bullet \)). In the region with lower frequencies, the signal intensity plateaued. At higher frequencies, it decreased. In addition, the spectral shape was similar to that obtained by the LISD microscope where the visible laser beam (wavelength = 532 nm) was used as a pump beam (Fig. 3, \( \bigcirc \)). It was verified that LISD measurements could also be carried out upon the application of the NIR laser beam.

The tolerance of biological samples to the exposure of the NIR laser beam was examined. As a test sample, a green-colored rice leaf, which strongly absorbs visible light for photosynthesis, was measured. The exposure to the visible laser beam left a hole with about 50 \( \mu \)m of diameter on the leaf surface and a burn was observed surrounding the hole (Fig. 4a). On the other hand, in the exposure to the NIR laser beam, no damage as seen with the visible laser was observed (Fig. 4b). It was shown that the application of the NIR laser beam led to the
decrease of photo damage in biological samples such as plants, which have strong absorption properties to visible lights.

It was investigated whether the NIR-LISD microscope allowed for discriminating the differences in power spectra between different regions in the same single cells. As a test cell, single rhizoids that had grown from the surface of a gemma collected from Marchantia polymorpha were measured (Fig. 5a). The single rhizoids are organs developed by the elongation of single cells from the gemma surface. In the single rhizoid, the power spectra for the subapical region containing its tip (apical region) and for the lateral region were measured with the NIR-LISD microscope. In the subapical region, the tip elongates. It has been shown by AFM measurements of pollen tubes that the elasticity in the tip growth regions of plants is lower than that in lateral regions due to immature cell walls in the tip regions.18,19 Figure 5b shows the power spectra for the subapical region and the lateral region in a single rhizoid. In both power spectra, the intensity plateaued at lower frequencies. At higher frequencies, the intensity decreased under the power-law with two exponents for the subapical region and a single exponent for the lateral region (solid lines in Fig. 5b). In the frequency range above ca. 3 × 10^4 Hz for the lateral region, the spectra were analyzed by a power-law with −2.0 ± 0.1 (mean ± s.d., n = 7) of exponent (dashed line in Fig. 5b), indicating that in this range of the frequencies surface displacements were buried into thermal fluctuation based on the linear elastic body theory (dashed line in Fig. 5b).20 It was confirmed that the subapical and lateral regions of the rhizoids were not damaged by the exposure to the pump and probe beams in the NIR-LISD measurements (Fig. 6).

The intensity in the plateau region in the subapical region was analyzed with the power-law with −2.0 ± 0.1 for discriminating the differences in power spectra between different regions in the same single cells. As a test cell, single rhizoids that had grown from the surface of a gemma collected from Marchantia polymorpha were measured (Fig. 5a). The single rhizoids are organs developed by the elongation of single cells from the gemma surface. In the single rhizoid, the power spectra for the subapical region containing its tip (apical region) and for the lateral region were measured with the NIR-LISD microscope. In the subapical region, the tip elongates. It has been shown by AFM measurements of pollen tubes that the elasticity in the tip growth regions of plants is lower than that in lateral regions due to immature cell walls in the tip regions.18,19 Figure 5b shows the power spectra for the subapical region and the lateral region in a single rhizoid. In both power spectra, the intensity plateaued at lower frequencies. At higher frequencies, the intensity decreased under the power-law with two exponents for the subapical region and a single exponent for the lateral region (solid lines in Fig. 5b). In the frequency range above ca. 3 × 10^4 Hz for the lateral region, the spectra were analyzed by a power-law with −2.0 ± 0.1 (mean ± s.d., n = 7) of exponent (dashed line in Fig. 5b), indicating that in this range of the frequencies surface displacements were buried into thermal fluctuation based on the linear elastic body theory (dashed line in Fig. 5b).20 It was confirmed that the subapical and lateral regions of the rhizoids were not damaged by the exposure to the pump and probe beams in the NIR-LISD measurements (Fig. 6).

The intensity in the plateau region in the subapical region was analyzed with the power-law with a single exponent, while that of the subapical region was analyzed based on two exponents. Here, the observation of multiple power-law exponents, namely the emergence of inflection points in the power spectra, is due to the existence of regions with different viscoelasticity in bodies. It was clarified that the subapical region comprises at least two different viscoelastic regions. Furthermore, the power spectrum of the subapical region showed that a power-law exponent (−1.7 ± 0.1 (mean ± s.d., n = 7)) was observed in the frequencies above 20 kHz, while in that of the lateral region, the displacement in the frequency region is buried into thermal fluctuation. The behavior indicated that the spectrum of the subapical region was able to follow the external force up to higher frequencies than that of the lateral region. In dynamic relaxation measurements of viscoelastic bodies, viscous resistance is increased at higher frequencies, and restoring forces due to elastic nature primarily affects bodies. In addition, with an increment in frequency of a periodical external force, more localized regions of the bodies are able to follow the force.21 From the power spectrum above 20 kHz in the subapical region, the local region in the subapical region is more elastic than the lateral region.

**Summary**

We developed an NIR-LISD microscope for the dynamic viscoelastic measurements of cell surfaces. It was shown that the use of the NIR laser beam led to the decrease of cell damage. The NIR-LISD microscope enabled us to measure the power spectra of single living plant cells (rhizoids). The differences in power spectra between the subapical and lateral regions of single rhizoids showed that the elasticity in the subapical region was lower than that in the lateral region, which was qualitatively consistent with the previous AFM results for tip growth region of pollen tubes. Furthermore, from the differences of the frequency dependence in the power spectra, it was clarified that the subapical region comprises at least two different viscoelastic regions. The NIR-LISD microscope is an advantageous tool for the dynamic viscoelastic measurements of cells, such as plant cells, that are susceptible to damage from ultraviolet or visible light.

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