Cytoskeletal Mechanics
Fast Fluorescence Microrheology

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Abstract — **The fluorescence laser tracking microrheometer (FLTM) is a novel instrument that proposes to assess the local, frequency-dependent mechanical properties of living cells with nanometer spatial sensitivity over a frequency range extending from 1 Hz to 50 kHz (1). This tutorial will familiarize researchers with the FLTM's theoretical principles and experimental routines, and will illustrate how chemically-induced cytoskeletal disruption is accompanied by reduced cellular stiffness and viscosity (as parameterized by $G*(\omega)$, the frequency-dependent complex shear modulus).**

I. MICRORHEOLOGY

TO gain insight in mechanotransduction and understand how the mechanical properties of cells are affected by their environmental cues, an assortment of microrheology schemes have been developed. They fall into two classes:

- active techniques (e.g. micropipette aspiration, AFM: atomic force microscopy, and magnetic manipulation), which involve mechanical force application to cells, and

- passive techniques (e.g. dynamic light scattering, and LTM: laser tracking microrheology), which examine the thermal fluctuations of probe particles (2-11).

A. Fluorescence laser tracking microrheometry (FLTM)

We have developed a fluorescence laser tracking micro-rheometer (FLTM) whose strengths are several folds.

1) The FLTM proposes to characterize the solid-like vs. liquid-like behavior of biomaterials using only microscopic amounts of sample, and without any active manipulation of the probed matter. FLTM takes advantage of the thermal energy of embedded colloids and relates their displacements to the viscoelastic properties of the surrounding medium: the more viscous and/or stiff the probe's microenvironment, the more confined its Brownian motion (12,13).

2) The FLTM's excellent speed (up to 50 kHz) and spatial resolution (~ 4 nm) make this apparatus well suited to the study of heterogeneous and dynamic biological systems.

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3) The extended frequency range (~ 0.5 Hz to 50 kHz) assayed by the FLTM allows the appraisal not only of the macroscopic viscoelasticity of molecular networks at long time scales (on the order of seconds), but also the fast bending and twisting fluctuations of single filaments at shorter time scales, which are fundamental for fast cytosketetal dynamics.

4) Relying on fluorescent probes (of characteristic spectral signatures) enables the targeting of cellular structures with molecular specificity.

B. Theory

The frequency-dependent complex shear modulus $G*(\omega)$ characterizes both the solid-like (through its real part $G'(\omega)$, the storage modulus) and the liquid-like (through its imaginary part $G''(\omega)$, the loss modulus) behaviors of a material. FLTM experimentally derives $G*(\omega)$ from a sphere's mean squared displacement, $MSD(\tau) = <\Delta r^2(\tau)> = <(r(t+\tau) - r(\tau))^2>_t$ (where $r(t)$ describes the particle's 2-dimensional trajectory, and τ corresponds to various lag times) by exploiting a generalized Stokes-Einstein relation (GSER)(14):

$$\left|G*(\omega)\right| \approx \frac{2k_B T}{3\pi a \left\langle \Delta r^2(1/\omega)\right\rangle \Gamma\left[1 + \alpha(\omega)\right]} \quad \text{(Eq. 1)}$$

$$G'(\omega) = \left|G*(\omega)\right| \cos(\pi\alpha(\omega)/2)$$
$$G''(\omega) = \left|G*(\omega)\right| \sin(\pi\alpha(\omega)/2), \quad \text{(Eq. 2)}$$

$$\alpha(\omega) \equiv \left. \frac{\partial \ln\left\langle \Delta r^2(\tau)\right\rangle}{\partial \ln \tau}\right|_{\tau=1/\omega} \quad \text{(Eq. 3)}$$

with k_B the Boltzmann constant, T the absolute temperature, a the radius of the probe particle, and Γ the gamma function.

II. FLTM EXPERIMENTS

A. Instrumentation: the FLTM setup

A schematic of the FLTM is presented in Figure 1: A laser beam[1] (532 nm, Verdi Nd:YVO$_4$, Coherent) is collimated through a custom light path and a 100X NA 1.30 oil objective[2] (Olympus) and illuminates a 100 μm x 100 μm area of sample positioned on the stage of an Olympus IX71 inverted microscope[3] and mounted on an *xy* piezoelectric nanopositioning system (Queensgate). The photons emitted by the fluorescent beads[4] (Molecular Probes) contained in the excitation volume are filtered by a dichroic mirror-barrier filter combination[5] (Chroma Technology) and detected after beam expansion by a quadrant photomultiplier tube[6] (PMT,
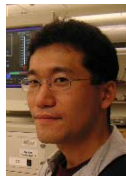
Hamamatsu). The total magnification of the system is of 2250 X, so that a 1-μm bead is finally projected on the 18mm x 18mm quadrant PMT as a disk of diameter 2.25 mm. Position signals are inferred at the PMT level from the difference in photocurrents between opposing pairs of quadrant elements, which are further amplified into digitized voltages by a 200 kHz 16-bit simultaneous 4-channel analog-to-digital converter (ADC, Strategic Test). For instance, the *x*-position of the probe particle is deduced from the signal ratio *RatioX* = (Ch0 + Ch2)/(Ch1 + Ch3) (Fig. 1).
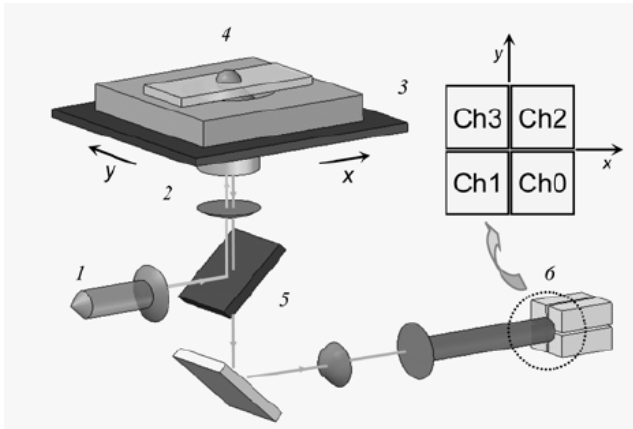
Fig. 1. Schematic diagram of the FLTM

B. Sample preparation: cell biology

NIH 3T3 fibroblasts were grown in standard 100mm x 20mm cell culture dishes (Corning) in DMEM (Cellgro®) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). Cells were cultured at 37°C in 5% CO_2. The day prior to the FLTM experiments, fibroblasts were plated on 35-mm glass-bottom cell culture dishes (MatTek) coated with collagen I (1 μg/cm², Cohesion Tech). On the day of the experiments, the cell confluency had reached about 60%. 1-μm orange fluorescent beads (Molecular Probes) coated with integrin antibodies were mixed to the growth media (at a concentration of 5 x 10⁵ microspheres/mL) and added to the plated cells for a period of 12 to 24 hours (for bead endocytosis). The influence of cytoskeletal components on cellular rheology was assessed by treating fibroblasts with the actin-disrupting drug cytochalasin D (10 μM) for 30 minutes before a final wash with culture media (Fig. 2).
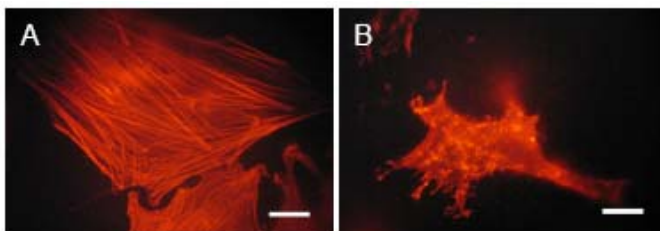

Fig. 2. Fluorescent labeling of F-actin (by Alexa Fluor® 568 phalloidin) in untreated (*A*) and cytochalasin-D treated (*B*) fibroblasts. Scale bars are 20 μm.

C. Data acquisition using LabVIEW (National Instruments)

- Position a single 1-μm bead at the center of the detector's field of view: `Scan Sample Shutter Background.vi`
  - fully automated command and coordination of the Queensgate xy stage (for raster-scanning of successively smaller areas) and the ADC acquisition card,
  - very low illumination power (< 5 x 10² W.m⁻²) to avoid photobleaching of the probe.

- Record the bead's fluorescent signal on the quadrant PMT using `Record Signal With Shutter.vi`
  - collection of 2^{18} = 262,144 data points at an ADC sampling rate of 200 kS/s.
  - high laser power (~ 5 x 10⁵ W.m⁻²): open iris at fiber input.

- Calibrate the FLTM with `Steps Shutter.vi`
  - translation of the sample along both detection axes (*x*, then *y*) and recording of the light distribution at each step.
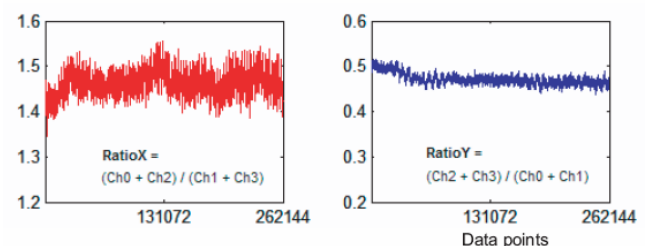
III. FLTM DATA PROCESSING (MATLAB)

A. From binary file to probe trajectory

- `GetSteps.m`: determine what *RatioX* and *RatioY* are at each bead position of the 10-nm-spaced calibration steps.
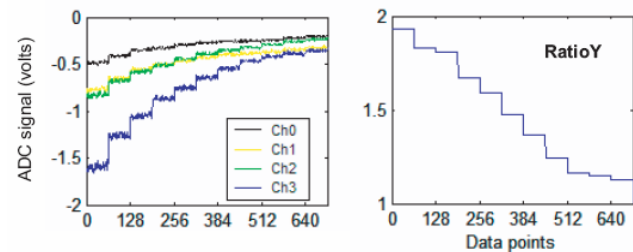
- `GetRatioXY.m`: calculate the bead's fluorescent signal's *RatioX* and *RatioY* at each data point (20-μs intervals).
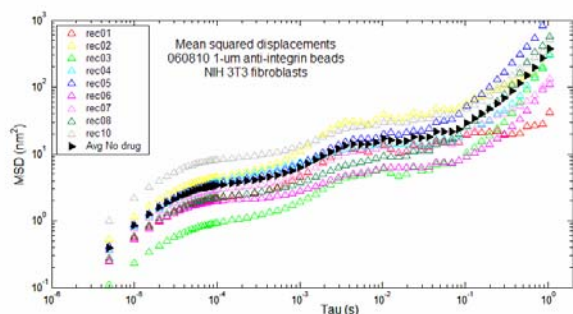

- `GetTrajBead.m`: for each time point, fit *RatioX* and *RatioY* to an (*x,y*) position doublet using the calibration curves.
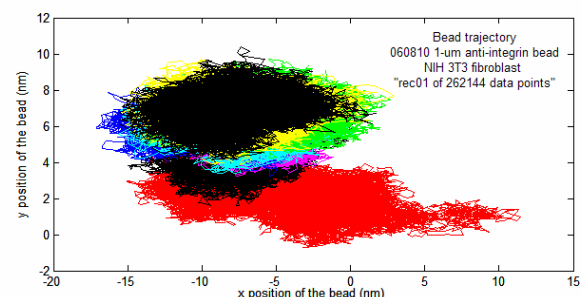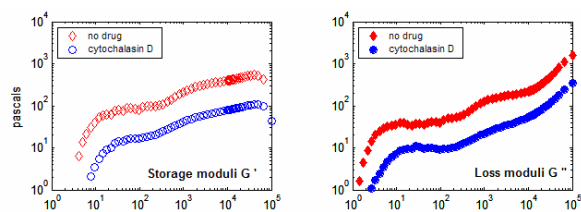
B. From bead trajectory to cell rheology

- `GetMSD.m`: calculate and save the particle's mean squared displacement from its 2D trajectory.

- `GetGstar.m`: compute the storage and loss moduli $G'(\omega)$ and $G''(\omega)$ from the probes' MSDs using Eq. 1-3.

IV. CONCLUSION

In conclusion, FLTM, benefiting from spatial and temporal sensitivities of 4 nm and 20 µs and sampling five decades of frequency (0.5 Hz to 50 kHz), is a passive microrheology technique well-suited to investigate biological problems, as illustrated by its aptitude to quantitatively characterize (i) the effect of cytoskeleton-disrupting drugs on cellular viscoelastic profiles, (ii) the difference between intracellular and surface fluorescent probes, and (iii) the impact of bead coating proteins on rheological results (1).

NOTES

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