



Chapter 11

Small Volume Microrheology to Evaluate Viscoelastic Properties of Nucleic Acid-Based Supra-Assemblies

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Abstract

Particle tracking (PT) microrheology is a passive microrheological approach that characterizes material properties of soft matter. Multicomponent materials with the ability to create extensive crosslinking, such as supra-assemblies, may exhibit a complex interplay of viscous and elastic properties with a substantial contribution of liquid phase still diffusing through the system. Microrheology analyzes the motion of microscopic beads immersed in a sample, making it possible to evaluate the rheological properties of biological supra-assemblies. This method requires only a small volume of the sample and a relatively simple, inexpensive experimental setup. The objective of this chapter is to describe the experimental procedures for the observation of particle motion, calibration of an optical setup for particle tracking, preparation of imaging chambers, and the use of image analysis software for particle tracking in viscoelastic nucleic acid-based supra-assemblies.

Key words Nucleic acids, Supra-assemblies, Viscoelastic properties, Microrheology, Particle tracking

1 Introduction

Rheology studies the flow and deformation of materials with complex viscoelastic properties under stress. Emulsions, suspensions, and supra-assemblies are a few examples of complex fluids. The flow of a material and its response to the applied stress can be predicted based on its rheological parameters. Furthermore, the rheological parameters offer a glimpse into the material's underlying microstructure. Traditional rheometers typically assess the frequency-dependent linear viscoelastic connection between strain and stress on the milliliter scale. Microrheology employs colloidal particles directly inserted in a soft material to measure rheological characteristics; the particles can be either externally driven by magnetic or optical tweezers (active microrheology) or rely on particle movement due to thermal motion (passive microrheology).

Particle tracking (PT) passive microrheology uses an inexpensive optical setup utilizing a research-grade microscope, a video camera, an objective lens, and a computer. This method can also operate with small volume chambers providing, thus, the means of studying samples where classical rheology experiments become prohibitively expensive. In passive PT microrheology, particle's motion is captured and further analyzed in terms of movement trajectories and particles' positions with time. This motion reports on the spatiotemporal rheological characteristics of the fluid sample, thus assessing how fluid influences particle's diffusive motion.

This chapter focuses on particle tracking passive microrheology (PT) technique. We discuss typical experimental setup, calibration procedures, chamber design, and data analysis. Furthermore, we demonstrate the method's utility on supra-assemblies made from mixtures of biotinylated DNA and streptavidin.

2 Materials

Materials for Supra-Assembly Synthesis

1. Double biotinylated DNA duplexes, with one biotin attached to the 5' end of each single-stranded complementary sequence (*see* Note 1).
2. Streptavidin.
3. Assembly buffer [final concentration of 89 mM tris-borate (pH 8.2), 50 mM KCl, and 2 mM MgCl₂].

Beads and Suspensions

All solutions were prepared using ultrapure water (~18 MΩ cm) and analytical-grade reagents.

Glycerol/Water Viscous Mixture for Calibration of the Experimental Setup

4. Glycerol.
5. SecureSeal hybridization chamber with a nominal volume of 30 μL.
6. Polystyrene beads with nominal diameter of 1 μm (*see* Note 2).
7. Glass coverslips 24 by 50 mm in size.

Particle Tracking

1. Olympus CX31 research-grade microscope (*see* Note 3).
2. Sentech CCD 4.0 MP camera with adjustable video capture rate (*see* Note 4).

3. PC with installed Sentech software for capturing videos (*see* Note 5).
4. 100X objective lens (*see* Note 6).
5. Fiji (ImageJ, <https://imagej.nih.gov/>) software with installed Mosaic plugin (*see* Note 7).

3 Methods

3.1 Supra-Assembly Synthesis

For the synthesis of supra-assemblies with different concentrations of DNA, the procedure is as follows (*see* Note 8):

1. Prepare and dissolve DNA duplex with biotinylated 5' strands at the desired concentration in assembly buffer.
2. Dilute streptavidin in assembly buffer in a 2:1 (duplex/streptavidin) molar ratio.
3. Add the streptavidin solution to the double biotinylated DNA duplex solution to prepare supra-assembly by rapidly pipetting up and down.
4. Vortex and centrifuge the final solution.
5. Incubate the solution at 37 °C for 30 min to complete binding.
6. For storage, keep the final solution at 4 °C.

3.2 Particle Tracking Setup and Its Calibration

The optical setup for observing the motion of the beads can be constructed around any research-grade microscope. The setup used in this study consists of CX31 Olympus upright microscope, 100× objective lens (air), and a variable scan speed camera Sentech CCD 4.0 MP, Fig. 1.

To calibrate the optical setup, the motion of the beads is analyzed in a set of glycerol/water mixtures of various glycerol-to-water ratios (as listed in Table 1—*see* Note 9) with known viscosity values, η .

Calibration includes establishing the actual distance in nanometers between pixels in the video. Tracking of the particle's motion returns trajectory measured in average distance traveled by the particle in pixels versus time, which is typically calculated using the time between two consecutive frames in the video. For two-dimensional or three-dimensional cases, the displacement is related to the diffusion coefficient simply by the following equation [1]:

$$\langle \Delta r^2 \rangle = 2pD\Delta t \quad \delta 1b$$

where p is dimensionality (for the optical setup and tracking procedure described here, $p = 2$ assuming the two-dimensional case of measurements performed in the x-y plane without consideration of the z-direction of particle's diffusion), r is the displacement in

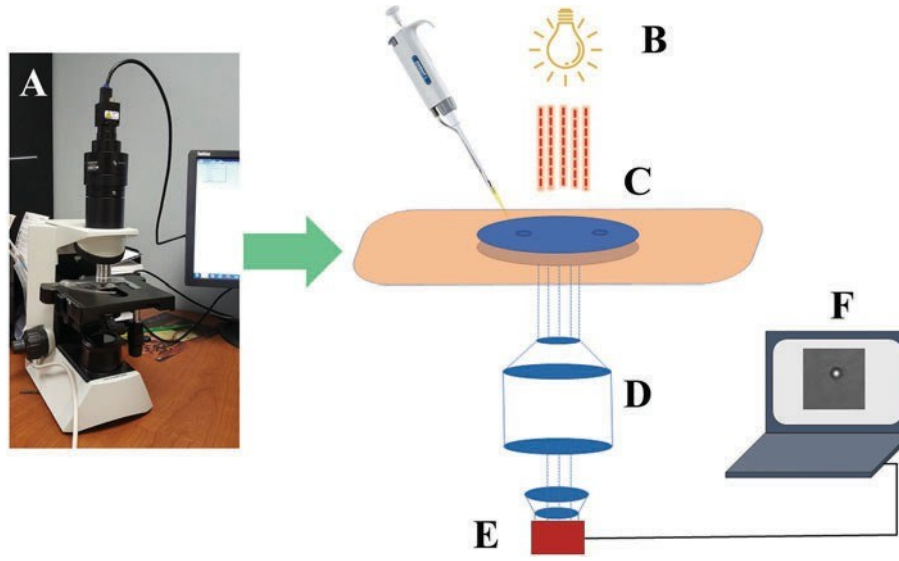


Fig. 1 Small volume microrheology setup with particle tracking capabilities: (a) assembled setup, (b) source of light, (c) small sample chamber, (d) objective lens, (e) variable scan rate camera, (f) computer with video capture and particle tracking analysis software

Table 1

Water/glycerol mixtures used for the calibration of the experimental setup

Glycerol to water ratio by weight	Viscosity, η (P·s)	Diffusion coefficient, D (m ² /s)
18.0% : 82.0%	1.66×10^{-3}	2.64×10^{-13}
32.2% : 67.8%	2.50×10^{-3}	1.75×10^{-13}
44.2% : 55.8%	4.65×10^{-3}	0.94×10^{-13}
64.5% : 35.5%	13.2×10^{-3}	3.32×10^{-14}

pixels in p dimensions, and Δt is the time interval. D is the diffusion coefficient related to the degree of particle's motion; particles with large diffusion coefficients fluctuate more and vice versa.

The Stokes-Einstein relationship for a spherical particle states that the fluctuation of a particle has the same origin as the dissipative frictional force the bead must work against to perturb the system in a particular direction:

$$D = \frac{k_B T}{6\pi\eta a} \quad \delta 2b$$

where $k_B T$ is thermal energy, η is the fluid's viscosity, and a is the particle's radius [1, 2]. Increasing the viscosity of the solution decreases the amplitude of particle's fluctuations which is explained by the increased friction experienced by the particle in a more viscous fluid. Known viscosity values for the mixtures presented in

Table 1 allow for establishing the relationships between known diffusion coefficient, D values, and the measured fluctuations of particle's position as a function of time. Pixel to nanometer coefficient, b , can be obtained using glycerol/water mixtures with known diffusion coefficients (Table 1). The average mean magnitude of the displacement (squared) between each frame measures how far the particle moves in time Δt (frame rate). The mean squared displacement of the particle then depends on the diffusion coefficient, D , and the time interval, (Δt) as:

$$D = \frac{\langle b^2 \Delta r^2 \rangle}{2\Delta t} \quad \text{83b}$$

Here, b is the coefficient that relates displacement, r , measured in pixels with known diffusion coefficients, D (in m^2/s^2). For the setup described in this study, the calibration yielded $b = 32.5 \times 10^{-9}$, indicating that the pixel-to-pixel distance is 32.5 nm. This coefficient, b , is used in all further calculations.

3.3 Bead Suspension Preparation and Video Acquisition

3.3.1 Glycerol/Water Mixtures

1. Weigh a moderate amount of glycerol into a vial.
2. Add the appropriate amount of water into the vial by measuring the volume of water with the pipette (recall the density of water is 1 g/mL) to make four mixtures with ratios listed in Table 1. Close the vial and shake it vigorously with a vortex mixer to ensure it is mixed uniformly.
3. Add 0.5 μL of stock bead solution (from the manufacturer's vial) of the desired size (1 μm in diameter size is recommended) into the vial with the sample to be observed.
4. Close the vial and shake it vigorously with a vortex mixer to ensure it is mixed uniformly with beads (*see* Note 10).
5. Place a self-adhesive hybridization chamber onto the center of a clean glass coverslip. GraceBio hybridization chambers are pre-equipped with a transparent top cover and two holes for convenient fluid sample deposition into the chamber.
6. Use the pipette to transfer roughly 30 μL of the prepared bead-mixed solution into the chamber, ensuring no bubbles are created.
7. Plug the holes with adhesive covers, keeping the solution from drying out.
8. Place the chamber onto the microscope stage. Move the objective lens away from the microscope stage before placing the slide onto the stage to avoid accidental damage.
9. Coverslip should be facing toward the objective lens. Turn on the light of the microscope.
10. Open the "Sentech" software to control the camera.

11. Move the objective slowly toward the chamber by rotating the focus adjusting knob.
12. Stop when beads become clearly visible in the video window of the “Sentech” software.
13. Record a 40-second video of the bead motion with 25 frames per second rate. These parameters are set in the “advanced options” tab of the “Sentech” software.
14. Save the video in *.avi format. Several beads are typically captured within one video.

3.4 Particle Tracking and Data Analysis

Fiji (ImageJ) software package can be used to perform a single particle tracking procedure using the Mosaic plugin (with a single Particle Tracking Analysis option—Fig. 2). Particle tracking uses brightness contrast above the background to extract accurate positions of the bead.

1. Open your video file in Fiji (ImageJ) software package.
2. In the pop-up window—unclick “use visual stack” and choose “Convert to Grayscale” (see Note 11).

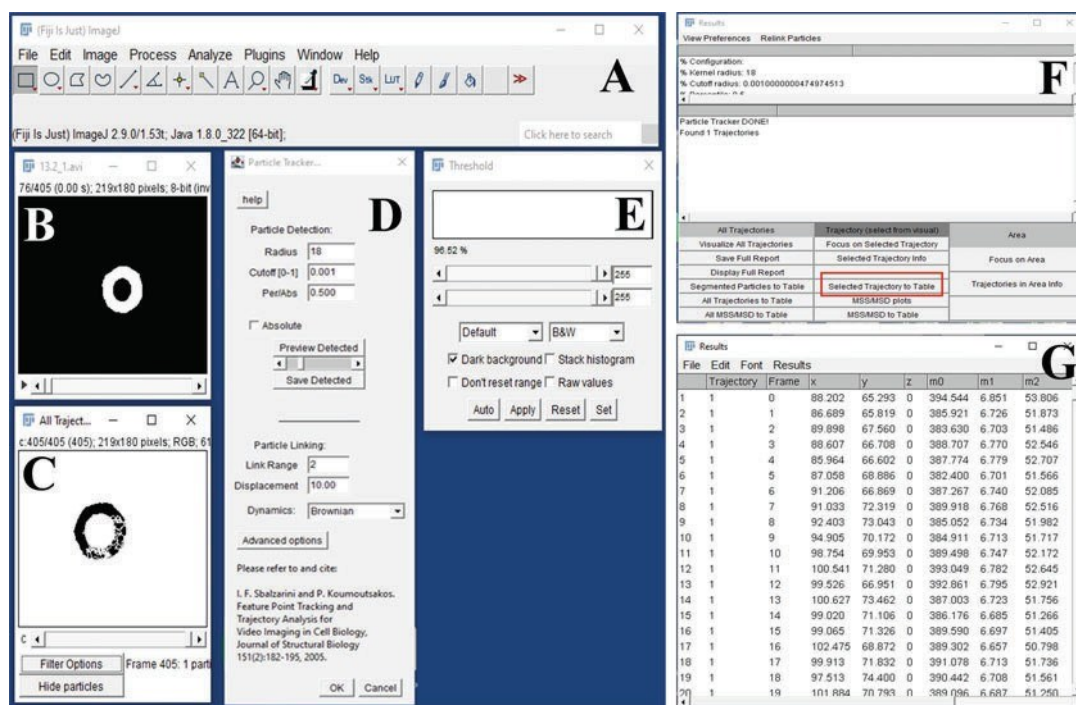


Fig. 2 Screenshot of the Fiji (ImageJ) and Mosaic (plugin) with input parameters for particle tracking analysis. (a) Main Fiji panel, (b) background corrected bead image, (C) tracked trajectory, (d) single particle tracking input parameters panel, (e) panel for defining of intensity for background correction, (f) results table, (g) saved table with trajectory data

3. Crop the video to include only the particles of interest (*see* Note 12).
4. Click “Image”/“Adjust”/“Threshold,” and check “Dark background.”
5. Click “Apply” with “Dark background” selected.
6. Click “ok” in the pop-up window with the “Default” method, “Dark” background, and “calculate the threshold for each image” option selected.
7. Navigate to the Mosaic plugin. “Plugins”/“Mosaic”/“Particle tracker 2D/3D.”
8. Choose the “No” option for the “Are these 3D data?” question.
9. In the Particle Tracker window—choose Radius ~ 20 and click preview detected.
10. Make sure the radius ring fits well the entire particle. Click “ok” (*see* Note 13).
11. When the tracking process is finished, and the “Results Table” window opens, press “Visualize all trajectories.” If several particles were visible within the field of view—there will be several trajectories tracked.
12. Pick a trajectory of interest by clicking on it and save “Selected Trajectory to the Table.”
13. The table contains trajectory data as bead position in x and y in pixels recorded for each frame. Use the conversion factor, b , established previously to convert the units from pixels to meters for both x and y .
14. To obtain rheological information for supra-assemblies, construct the ensemble-averaged mean-squared displacement (MSD), $\langle \Delta r^2 \rangle$, as a function of lag time, τ , from the unit corrected bead trajectories. $\Delta r^2(i) = \Delta x^2(i) + \Delta y^2(i)$ and $\langle \Delta r^2(\tau) \rangle = \langle \sum (r_{i+n} - r_i)^2 \rangle$, where n is $n = 1, 2, 3 \dots N-10$ and N is the total number of frames in the video (*see* Note 14).
15. MSD can be related to the diffusion coefficient, D , using the following equation [3]:

$$\langle \Delta r^2(\tau) \rangle = 2pD\tau^a \quad 04p$$

16. Plots of MSD vs. τ measured for supra-assemblies of different concentrations, 1, 5, and 7 μM , are shown in Fig. 3a–c, respectively. For comparison, Fig. 3d–g show MSD vs. τ plots for glycerol/water mixtures of the following viscosity values 1.66 mP s, 2.5 mP s, 4.65 mP s, and 13.2 mP s, respectively.

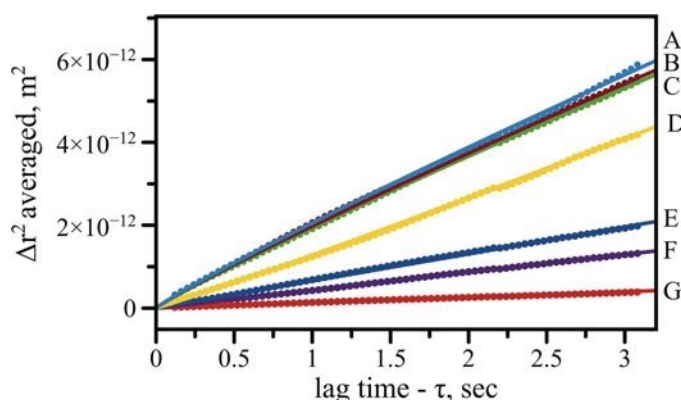


Fig. 3 MSD vs. lag time (τ) plots. (a) 1 μ M supra-assembly, (b) 5 μ M supra-assembly, (c) 7 μ M supra-assembly, (d) 1.66 mP·s glycerol/water mixture, (e) 2.5 mP·s glycerol/water mixture, (f) 4.65 mP·s glycerol/water mixture, (g) 13.2 mP·s glycerol/water mixture

Table 2
Parameters of the fit for MSD vs. τ plots

Sample	Lag time power, α	Diffusion coefficient, D (m ² /s)
A, 1 μ M H-gel	0.92	5.14×10^{-13}
B, 5 μ M H-gel	0.92	4.95×10^{-13}
C, 7 μ M H-gel	0.93	4.75×10^{-13}
D, 1.66 mP s	1.02	2.84×10^{-13}
E, 2.5 mP s	1.00	1.64×10^{-13}
F, 4.65 mP s	1.00	1.08×10^{-13}
G, 13.2 mP s	1.00	3.30×10^{-14}

17. The plots are fitted using Eq. 4 while setting $p = 2$ (2D case) with D and α as fitting parameters, Table 2.
18. Power of the lag time, α , identifies the state of the material. $\alpha = 1$ corresponds to freely diffusing particles in a purely viscous environment, such as glycerol/water mixtures representing Newtonian fluids. For $0 < \alpha < 1$, particles move in a viscoelastic fluid, and the material's structure restricts their motion. Lower values of α correspond to a greater restriction of the particle's motion, becoming completely arrested when $\alpha \rightarrow 0$ (see Note 15).

4 Notes

1. The sequences used are listed in the supporting information of Chandler et al. (2021) as DNA for Sense_12_Biotin and DNA for Antisense_12_Biotin. Any biotinylated sequences should work with this procedure [4].
2. Polystyrene microspheres are a good choice of beads since the density of polystyrene is similar to the density of water which allows these beads to be easily suspended in aqueous solutions without settling due to gravity. Also, the beads with no surface modification are preferred to avoid unnecessary surface-solute interactions so that it does not interfere with the purely diffusive motion of the beads.
3. Any other research-grade microscope, upright or inverted, would also be suitable for setting up a particle tracking optical system. We have also successfully used Olympus IX-71 inverted microscope with the same set of camera and objective lens.
4. Adjustable rate of the camera for video capturing provides more flexibility for a researcher to adjust how fast the motion of the beads is captured. It is beneficial for larger bead sizes and more viscous samples.
5. Captured videos may be very large; make sure the PC used has enough memory to store data.
6. We use high power 100× objective lens as part of the optical setup to provide better tracking capabilities. However, other objective lenses could be used; for example, 40× magnification is also suitable.
7. Mosaic plugin installed within the Fiji (ImageJ) software allows particle tracking to be performed in an easy and intuitive routine flow (<http://mosaic.mpicbg.de/?q=downloads/imagejor>, <https://sbalzarinilab.org/?q=downloads/imagej>) [5]. There are other plugins for ImageJ capable of similar particle tracking capabilities, for example, PTA (Particle Tracking and Analysis).
8. This procedure follows the DNA Duplex + QD assembly, but using streptavidin in place of quantum dots [4].
9. Glycerol is highly viscous and cannot be measured precisely using the pipette as it will adhere to both the inside and outside of the pipette tip. To calculate the amount of glycerol, it is better to use a chemical balance to weigh the amount of glycerol and use a pipette to add water.
10. The size of the bead is an important parameter to consider, as the supra-assembly's microstructure can impact the bead's mobility. For example, supra-assemblies presented herein,

while compliant for beads up to 2 μm in diameter, tend to arrest the motion of larger beads. Concentration-dependent arresting efficiency suggests complex microstructures of the supra-assemblies. The concentration of the beads and the manufacturer's specifications may vary from batch to batch. Adjust the amount of added bead solution based on the desired amount of beads in the video's field of view.

11. "Convert to grayscale" option allows the program to calculate the brightness of the bead correctly. If not chosen, the background will not be calculated properly.
12. Including only particles of interest reduces the time required to track a trajectory. Smaller cropped windows provide faster analysis. Scroll to the end of the video to ensure the particle of interest always stays within the cropped window.
13. Scroll through the entire video to check that the radius ~ 20 fits well throughout the entire length of the video. Sometimes, the particles also diffuse in and out of focus, and the particle size changes; if so, adjust the radius to fit the particle better. Also, due to in- and out-of-focus diffusion, the program may lose track of the particle for several frames. In this case, the tracking can be improved by selecting more than 2 (default) frames and larger than 10 (default) distances to link (Fig. 2d).
14. Limiting $\langle \Delta r^2(\tau) \rangle$ calculation to $N-10$ frames rather than N provides more reliable averaging of the last values of Δr^2 with at least 10 values to average.
15. While supra-assemblies presented here show nice behavior of the MSD vs. τ plots, one has to keep in mind that more complex systems can be analyzed with the passive microrheology technique. More curved plots can be analyzed by including higher-order terms in the analysis of $\langle \Delta r^2(\tau) \rangle$ [3]. Another rheological parameter easily extractable from the measured MSDs is the creep compliance, $J_{\text{creep}} = \frac{3\pi r}{4k_B T}$ [3, 6], which in the case of supra-assemblies presented here returned very similar linear dependences suggesting linear increase of the strain rate for a constant applied stress for these materials [7].

Acknowledgments

Research reported in this publication was supported by the National Science Foundation, Division of Material Research, Award Numbers 2203946 (to K.A.A.) and 2204027 (to A.V.K.), and the National Institute of General Medical Sciences of the National Institutes of Health under Award Number R35GM139587 (to K.A.A.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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