Fluorescence microscopy techniques for characterizing the microscale mechanical response of entangled actin networks

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Abstract

Actin filaments are semiflexible polymers that display complex viscoelastic properties when entangled in networks. In order to characterize the molecular-level physical and mechanical properties of entangled actin networks it is important to know the in-network length distribution and the response of entangled filaments to local forcing. Here we describe two single-molecule microscopy protocols developed to investigate these properties. Using confocal fluorescence microscopy and ImageJ image analysis we have developed a protocol to accurately measure the in-network actin length distribution. To characterize the deformation of actin filaments in response to perturbation, we trap micron size beads embedded in the network with optical tweezers and propagate the beads through the entangled filaments while simultaneously recording images of fluorescent-labeled filaments in the network. A sparse number of labeled filaments dispersed throughout the network allow us to visualize the movement of individual filaments during perturbation. Analysis of images taken during forcing is carried out using a combination of vector mapping and skeletonization techniques to directly reveal the deformation and subsequent relaxation modes induced in entangled actin filaments by microscale strains. We also determine the dependence of deformation modes on the relative filament position relative to the strain.

Actin filament and network deformation in response to forcing

Network Preparation

For network displacement analysis we form partially labeled actin filaments and track the movement of the filaments as beads are translated through the network. To obtain partial labeling within the network we first polymerize Alexa 568 dyed filaments at 5 µM. Next we shear the filaments using a 26 gauge hamilton syringe. Then 5 µM dark actin is added to the sheared sample in order to anneal partially labeled filaments. After the annealing process, the sample is diluted 400 fold and added to a sample of 11.9 µM dark actin that will form a 0.5 mg/ml network around the partially labeled filaments. Finally 4.5 µm BSA coated beads are added to the sample and it is flowed into a flow cell.

Forcing Experiments

The beads are trapped using a 1064 nm Nd:YAG fiber laser focused by a 60x objective. A piezoelectric mirror is used to move the trapped bead through the network. Each displacement experiment is split into five phases; the phases can be seen in the schematic above. The first phase keeps the trap fixed to allow the bead to equilibrate within the network. After about ten seconds the trap is moved at constant velocity 10 µm. The third phase is a 15 second relaxation period. The bead is then moved 10 µm back to its original position in phase four. The last phase is another 15 second relaxation. During these steps the network is being recorded via fluorescence microscopy. Figure 1 shows a time-lapse of this process.

Figure 1: Filament Response to Forcing.

Using an Olympus IX70 microscope images are taken at a rate of 0.4 seconds per frame. A 4.5 µm bead propagates through an actin network containing partially labeled filaments. The initial and final positions of the bead are circled permanently in white, the blue arrow indicates where the bead is when it is in motion, and the purple circle highlights a single filament with multiple label markers that is displaced during the strain. Here you can watch how filaments respond to the five phases of motion.

Figure 2: Tracking Filament Motion.

Figure 3: Filament Velocity.

Graph of the net velocity of the filaments during the first three phases of the experiments. Each data was gathered from filament tracking plots. Each color represents a different distance from the bead’s path as indicated in bead diameters from the movement track.

Measuring length distributions and homogeneity in networks

Sample Preparation

The actin used for the length distribution analysis was polymerized at a concentration of 0.5 mg/ml, labeled with 550 molar ratio Alexa Fluor 488 dye, and stabilized with 20% molar ratio dark phallloid. The stabilized actin networks were diluted 500 fold in F-buffer then flowed into the prepared channels. Coverslips were coated with 0.1% nitrocellulose so that the filaments would stick to the coverslip and stay in one place for imaging. Flow channels were made with the coverslips and double sided sticky tape.

Measuring the Distribution

Images of the slides were captured using a Nikon A1R confocal microscope. To ensure that the filaments were sticking to the surface, three minute long image stacks with five second intervals were captured. Using ImageJ we manually select each filament to be measured. All of the images taken had a resolution of 0.412 µm/pixel or 0.206 µm/pixel. Using measure analysis tool in ImageJ we measured pixel length of each filament in the image stacks.

Figure 3: Filament Velocity.

(a) An average of a three minute long video visualizing actin filaments stuck to a coverslip surface in order to be measured. The magnified portion of the image shows the length measurement technique used on ImageJ. (b) A composite image of the average and maximum intensity extracted from the same video as (a). This shows which filaments are completely stuck to the coverslip surface and which are partially stuck with tails in solution. We can look at this figure to determine which filaments to measure. A 15 µm scale can be seen in the top right of each image.

Figure 4: Measurement Technique.

Figure 5: Normalized Distribution of Optically Measured Filament Lengths.

A histogram and fit curve of the filament length distribution was created using MATLAB. The distribution shows the measured lengths of 1545 actin filaments polymerized at 0.5 mg/ml. The distribution is well fit to a gamma distribution with a scale parameter of 2.22 and a scale of 4.73. The mean length is 6.85 µm with a standard deviation of 3.15 µm. The measurements were collected using confocal fluorescence microscopy as discussed above.

Conclusion

The methods illustrated in this presentation are two steps necessary to characterizing the properties of entangled actin networks. Being able to accurately find the length distribution of the networks is important because length distributions of biopolymer networks are intimately linked to the complex viscoelastic behaviors exhibited within the networks. The length distribution collates with several properties of the network; it can tell us information about the potential growth of the network, the viscosity of the network, diffusion rates, and alignment within the network. It is also important to characterize molecular deformation leading to complex network responses. The protocol we have developed visually reveals the deformation and relaxation modes within the entangled actin networks. Imaging strain and relaxation of the networks reveals insights into the complex nonlinear dynamics exhibited by entangled semiflexible polymers.