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Supplementary Information

Integration of Raman Tweezers and Machine Learning for Label-Free Single-Cell Characterization of Endometriosis Cells

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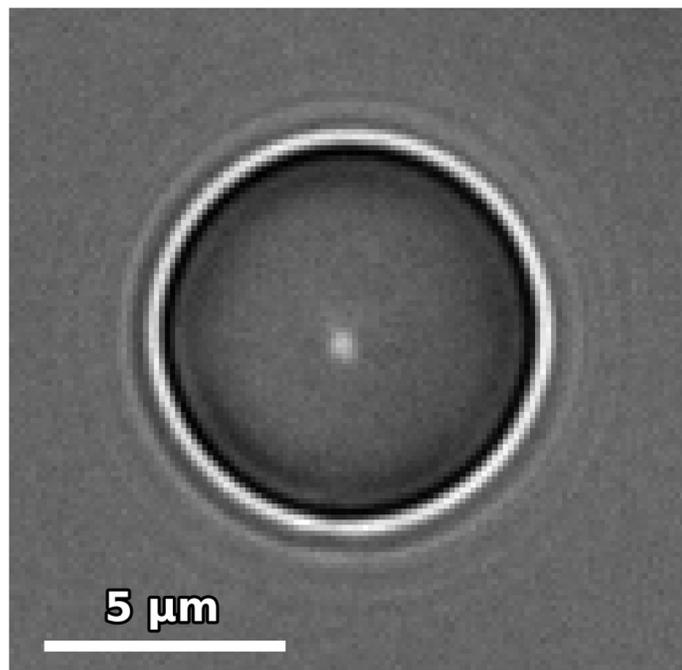
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Trap Stiffness by Power Spectral Density Analysis

In this study, we applied Power Spectral Density (PSD) analysis to quantitatively assess the stiffness of an optical trap using a 6.78 μm polystyrene bead. The motion of the trapped bead was recorded using a high-speed video microscopy system at a sampling frequency of 77.34 Hz (exposure time = 0.005 seconds, number in kinetics series = 10000). This acquisition rate was selected to capture the thermal fluctuations of the bead with sufficient temporal resolution.

The time-lapse sequence was saved in TIFF format of image stack. Each frame was normalized to 8-bit grayscale, followed by histogram equalization to enhance contrast. This is based on the fact that each pixel is represented by one byte and can take any integer value from 0 to 255. Next, adaptive Gaussian thresholding was applied to segment the bead from the background [1]. In the image, the beads being trapped by the optical tweezers appear as uneven illumination with more brightness at the edges and the center (Figure S1). Adaptive Gaussian thresholding works by looking at each small patch of the image, computing a local brightness level (using a Gaussian-weighted average of the pixels in that patch), then subtracting a small constant (here we choosed the value of 2) to set a threshold. Any pixel brighter than its local threshold is turned “on” (white) and everything else “off” (black). Because the threshold is computed separately for each region, it automatically adapts to uneven illumination. The result would be a binary mask with beads as bright, roughly circular regions.



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Figure S1. Focus image of a 6.78 μm polystyrene bead being trapped by the optical tweezers

49 From that, circular contours corresponding to individual beads were extracted to determine
50 the center coordinates (X, Y) of each bead in every frame, along with the associated frame number.

51 As a Nikon 60× objective lens was used, the physical size of each pixel in the captured
52 images was approximately 0.167 μm, calculated from a camera pixel size of 10 μm divided by the
53 magnification (10 μm/60×). The extracted bead positions were converted to physical units (μm)
54 and saved to a CSV file.

55 The position fluctuations of the trapped microsphere over time in the CSV file are then
56 Fourier transformed to obtain the power spectrum, which follows a characteristic Lorentzian
57 profile [2]:

$$P(f) = D/(2\pi^2(f_c^2 + f^2))$$

58
59 Where:

- 60 • P(f) is the power spectral density as a function of Fourier frequency f (Hz)
- 61 • D is the bead's diffusion coefficient, given by $D = k_B T / \gamma$
- 62 • k_B is the Boltzmann constant ($1.38 \times 10^{-23} \text{ J K}^{-1}$)
- 63 • T is the absolute temperature, in our case is the room temperature (293.15 K)
- 64 • f_c is the corner frequency
- 65 • $\gamma = 6\pi\eta a$ is the particle's friction coefficient (computed below)

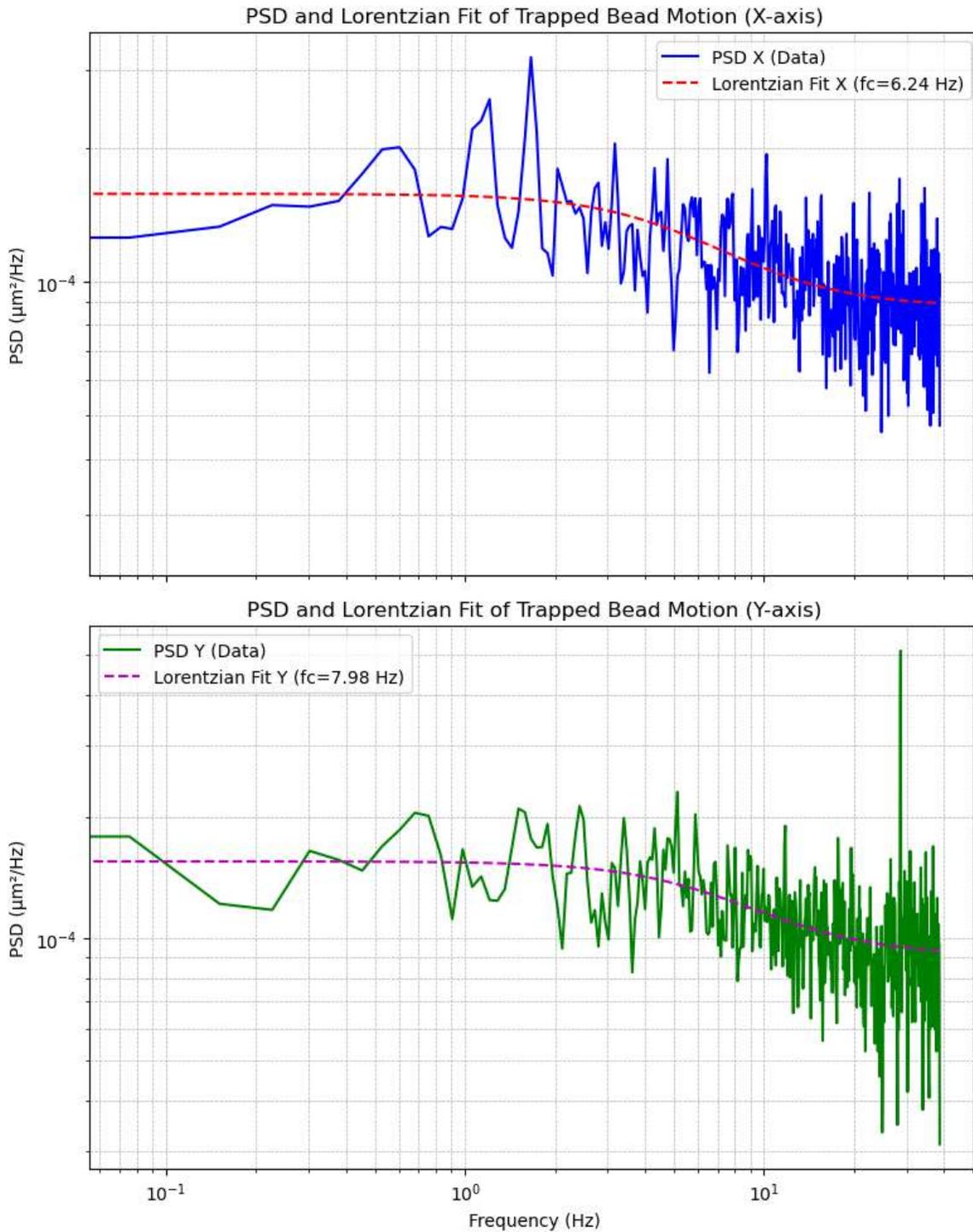
66 The corner frequency (f_c) appears as the frequency at which the power spectrum drops to
67 half of its maximum value. This parameter is directly proportional to the trap stiffness through the
68 relation [2]:

$$\kappa = 2\pi\gamma f_c$$

69
70 Where:

- 71 • κ is the trap stiffness (in pN/μm)
- 72 • $\gamma = 6\pi\eta a$ is the particle's friction coefficient
- 73 • η is the medium viscosity, as for PBS is 0.89 mPa·s
- 74 • a is the particle radius ($6.78 \mu\text{m}/2 = 3.39 \mu\text{m}$)

75 Thus, we can estimate the trap stiffness of optical tweezers using the f_c yielded from the
76 Lorentzian fitting. In our study, we calculated the trap stiffness in for both X and Y coordinates.



77
 78 **Figure S2.** PSD analysis with Lorentzian fit of trapped bead motion based on X and Y axis (blue
 79 and green, respectively)

80 The fitting procedure yielded stiffness values of 2.23 pN/μm and 2.85 pN/μm along the X-
 81 and Y-axes, respectively, demonstrating the isotropic nature of the trap (Figure S2).

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