

PHYS 332 Advanced Physics Laboratory

Laboratory Notebook

Experiment: Microscopy and Cell Motility

Lab Period 1: Brownian Motion of Polystyrene Spheres

Date	February 3, 2026
Time	1:30 PM - 4:30 PM
Lab Partners	Ahi (Author), Nathan
Workstation	Biophysics Lab, Station 2
Microscope	Carl Zeiss Axioskop 2 Plus
Camera	FLIR BlackFly S (1288 × 964 pixels)

Session Goals

The specific, measurable goals for this lab session are:

1. Learn operation of upright bright-field microscope and achieve proper Köhler illumination
2. Calibrate the 100× oil-immersion objective using stage micrometer ($\pm 2\%$ uncertainty target)
3. Prepare diluted sample of 1 μm polystyrene spheres with ~20 beads in field of view
4. Capture 60-second video of Brownian motion at 30 fps (1800 frames minimum)
5. Extract particle trajectories and compute diffusion coefficient D
6. Compare measured D to Stokes-Einstein prediction within stated uncertainties

Pre-Lab Preparation

Completed: February 2, 2026 (day before lab)

Pre-Lab Question 1: Bead Dilution Calculation

Given parameters:

- Calibration factor: 100 nm/pixel
- Camera field of view: 1288 × 964 pixels
- Depth of field: ~5 μm
- Bead diameter: $d = 1 \mu\text{m}$
- Polystyrene density: $\rho = 1.05 \text{ g/mL}$
- Stock concentration: 0.5 wt% (= 5 mg/mL)
- Target: ~20 beads in field of view

Calculation:

Step 1: Convert field of view to physical dimensions

FOV width = 1288 pixels × 100 nm/pixel = 128.8 μm

FOV height = 964 pixels × 100 nm/pixel = 96.4 μm

Step 2: Calculate observation volume

$$V_{\text{obs}} = 128.8 \mu\text{m} \times 96.4 \mu\text{m} \times 5 \mu\text{m} = 62,080 \mu\text{m}^3 = 6.21 \times 10^{-8} \text{ mL}$$

Step 3: Calculate volume and mass of single bead

$$V_{\text{bead}} = (4/3)\pi r^3 = (4/3)\pi(0.5 \mu\text{m})^3 = 5.24 \times 10^{-13} \text{ mL}$$

$$m_{\text{bead}} = \rho V = 1.05 \text{ g/mL} \times 5.24 \times 10^{-13} \text{ mL} = 5.50 \times 10^{-13} \text{ g}$$

Step 4: Calculate required number density for 20 beads

$$n = 20 \text{ beads} / 6.21 \times 10^{-8} \text{ mL} = 3.22 \times 10^8 \text{ beads/mL}$$

Step 5: Calculate required mass concentration

$$c_{\text{required}} = n \times m_{\text{bead}} = 3.22 \times 10^8 \times 5.50 \times 10^{-13} \text{ g} = 1.77 \times 10^{-4} \text{ g/mL}$$

Step 6: Calculate dilution factor

$$\text{Dilution factor} = c_{\text{stock}} / c_{\text{required}} = (5 \times 10^{-3} \text{ g/mL}) / (1.77 \times 10^{-4} \text{ g/mL}) \approx 28$$

CONCLUSION: Dilute stock solution approximately 1:30 (add 1 μL stock to 29 μL water).
Adjust empirically based on observed bead count.

Pre-Lab Question 2: Expected Diffusion Coefficient

Stokes-Einstein Relation:

$$D = k_B T / (6\pi\eta r)$$

Parameters at room temperature (T = 295 K ≈ 22°C):

- $k_B = 1.381 \times 10^{-23} \text{ J/K}$
- η (water at 22°C) = $9.55 \times 10^{-4} \text{ Pa}\cdot\text{s}$
- $r = 0.5 \mu\text{m} = 5 \times 10^{-7} \text{ m}$

Calculation:

$$D = (1.381 \times 10^{-23} \text{ J/K})(295 \text{ K}) / [6\pi(9.55 \times 10^{-4} \text{ Pa}\cdot\text{s})(5 \times 10^{-7} \text{ m})]$$

$$D = 4.07 \times 10^{-21} / 9.00 \times 10^{-9}$$

$$D = 4.53 \times 10^{-13} \text{ m}^2/\text{s} = 0.453 \mu\text{m}^2/\text{s}$$

CONCLUSION: Expected diffusion coefficient: $D = 0.45 \pm 0.02 \mu\text{m}^2/\text{s}$ (uncertainty from temperature $\pm 1^\circ\text{C}$)

Pre-Lab Question 3: Expected RMS Displacement

For 2D diffusion, the mean-squared displacement is:

$$\langle r^2 \rangle = 4D\Delta t$$

For Δt = 1/30 s (single frame interval at 30 fps):

$$\langle r^2 \rangle = 4 \times 0.453 \mu\text{m}^2/\text{s} \times (1/30 \text{ s}) = 0.0604 \mu\text{m}^2$$

$$r_{\text{rms}} = \sqrt{0.0604} = 0.246 \mu\text{m} = 246 \text{ nm} \approx 2.5 \text{ pixels}$$

For $\Delta t = 1 \text{ s}$:

$$\langle r^2 \rangle = 4 \times 0.453 \mu\text{m}^2/\text{s} \times 1 \text{ s} = 1.81 \mu\text{m}^2$$

$$r_{\text{rms}} = \sqrt{1.81} = 1.35 \mu\text{m} \approx 13.5 \text{ pixels}$$

CONCLUSION: Per frame ($\sim 33 \text{ ms}$): expect ~ 2.5 pixel displacement. Per second: expect ~ 13.5 pixel displacement. Beads should show visible random motion.

Pre-Lab Question 4: Gaussian Distribution of Displacements

The displacement components Δx and Δy in time Δt follow Gaussian distributions:

$$P(\Delta x) = (1/\sqrt{4\pi D\Delta t}) \exp(-\Delta x^2 / 4D\Delta t)$$

The variance of Δx (and Δy) is:

$$\sigma^2 = \langle \Delta x^2 \rangle = 2D\Delta t$$

For $\Delta t = 1/30 \text{ s}$:

$$\sigma = \sqrt{2 \times 0.453 \times 1/30} = 0.174 \mu\text{m} = 1.74 \text{ pixels}$$

CONCLUSION: Histogram of Δx should be Gaussian with mean 0 and standard deviation $\sigma \approx 1.7$ pixels. The diffusion coefficient can be extracted from $D = \sigma^2/(2\Delta t)$.

Pre-Lab Question 5: Effect of Temperature on Diffusion

The Stokes-Einstein relation shows D depends on both T and $\eta(T)$:

$$D \propto T/\eta(T)$$

Since viscosity decreases with temperature (approximately exponentially), and $D \propto T/\eta$:

- Both factors (increasing T , decreasing η) cause D to increase with temperature.
- The viscosity effect dominates: a 1°C change alters η by $\sim 2\%$, while T changes by $\sim 0.3\%$.

Practical implication:

Record the room temperature during measurements. A 5°C uncertainty in temperature leads to $\sim 10\%$ uncertainty in the predicted D value.

CONCLUSION: Temperature control is important. Measure and record room temperature; expect $\sim 2\text{-}3\%$ change in D per $^\circ\text{C}$ near room temperature.

Experimental Work

Session Start: 1:30 PM

1. Equipment Setup and Microscope Familiarization

Time: 1:30 PM - 2:00 PM

Equipment List:

Item	Details/Settings
Microscope	Carl Zeiss Axioskop 2 Plus, upright configuration
Objectives	10× (NA 0.25), 40× (NA 0.65), 100× oil (NA 1.25)
Camera	FLIR BlackFly S BFS-U3-13Y3M, 1288×964 px, monochrome
Software	Vision Assistant (LabVIEW), ImageJ 1.54f
Immersion oil	Zeiss Immersol 518 F, n = 1.518
Stage micrometer	Graticules Ltd., 1 mm / 100 div (10 µm/div)
Micropipettes	Eppendorf, 2-20 µL and 20-200 µL
Thermometer	Digital, ±0.5°C accuracy

Variables Definition:

Variable Type	Variable	Description
Independent	Time t	Frame number × frame interval
Dependent	x(t), y(t)	Bead centroid position in pixels
Dependent	Δx, Δy	Frame-to-frame displacement
Control	Temperature	Room temperature: 22.3 ± 0.5°C
Control	Bead size	1 µm diameter polystyrene
Control	Medium	Deionized water
Control	Frame rate	30 fps ($\Delta t = 33.3$ ms)

Köhler Illumination Setup Procedure:

(Following Protocol: Microscope Setup)

7. 1. Turned on illumination lamp and allowed 5 min warm-up
8. 2. Placed blank slide on stage, focused with 10× objective
9. 3. Closed field diaphragm until edges visible in FOV
10. 4. Adjusted condenser height until field diaphragm edges sharp
11. 5. Centered condenser using centering screws
12. 6. Opened field diaphragm to just outside FOV
13. 7. Adjusted aperture diaphragm to ~70% of objective NA for optimal contrast

Note: Field diaphragm controls illumination area; aperture diaphragm controls NA (and thus resolution/contrast tradeoff).

2. Camera Calibration with Stage Micrometer

Time: 2:00 PM - 2:30 PM

Procedure:

14. 1. Placed stage micrometer on microscope stage
15. 2. Focused on micrometer lines using 10× objective first
16. 3. Applied small drop of immersion oil to coverslip
17. 4. Rotated 100× oil objective into position (parfocal - minimal refocus needed)
18. 5. Launched Vision Assistant, set exposure to 10 ms, gain to 0 dB
19. 6. Captured image of micrometer scale bars
20. 7. Opened image in ImageJ for calibration measurement

Calibration Data:

File: Calibration/2026-02-03_calibration_100x.tif

Used ImageJ line profile tool (width = 5 pixels for averaging) to measure distance between micrometer divisions:

Measurement	Divisions	Known Distance (μm)	Pixels
1	10	100	1024
2	10	100	1021
3	10	100	1026
4	10	100	1022
5	10	100	1025

Analysis:

Mean pixel count: $(1024 + 1021 + 1026 + 1022 + 1025) / 5 = 1023.6$ pixels

Standard deviation: $\sigma = 2.1$ pixels

Standard error of mean: $\sigma/\sqrt{5} = 0.94$ pixels

Calibration factor calculation:

Scale = 100 μm / 1023.6 pixels = 0.0977 μm/pixel = 97.7 nm/pixel

Uncertainty propagation:

$$\delta(\text{Scale}) = \text{Scale} \times (\delta\text{pixels} / \text{pixels}) = 97.7 \times (0.94 / 1023.6) = 0.09 \text{ nm/pixel}$$

Systematic uncertainty: micrometer accuracy specified as ±0.5% = ±0.5 nm/pixel

Combined uncertainty (quadrature): $\sqrt{(0.09^2 + 0.5^2)} \approx 0.5 \text{ nm/pixel}$

CONCLUSION: Calibration: $97.7 \pm 0.5 \text{ nm/pixel}$ (0.5% relative uncertainty). Field of view: 126 μm × 94 μm.

3. Sample Preparation

Time: 2:30 PM - 2:50 PM

Materials:

- Polystyrene microspheres: Polysciences Inc., 1.0 μm diameter, Cat. #07310

- Stock concentration: 0.5 wt% (5 mg/mL)
- Deionized water (MilliQ, 18.2 MΩ·cm)
- Glass microscope slides (Fisher Scientific)
- #1 coverslips (22 × 22 mm, 0.13-0.17 mm thick)
- Parafilm spacers (~100 µm thick)

Dilution procedure:

Based on pre-lab calculation, target dilution ~1:30

21. 1. Vortexed stock solution for 30 seconds to disperse aggregates
22. 2. Pipetted 2 µL of stock into microcentrifuge tube
23. 3. Added 58 µL of DI water (1:30 dilution)
24. 4. Gently mixed by pipetting up and down 10 times

Chamber preparation:

(Following Protocol: Making Sample Chambers - thick chamber method)

25. 1. Cut two strips of Parafilm (~3 mm wide, ~20 mm long)
26. 2. Placed strips parallel on glass slide, ~15 mm apart
27. 3. Heated on heat block at 70°C for 10 s to seal Parafilm to slide
28. 4. Pipetted 10 µL diluted bead solution between Parafilm strips
29. 5. Gently lowered coverslip onto Parafilm strips
30. 6. Sealed edges with nail polish (quick-dry, 2 min)

Quality check:

Initial observation with 40× objective showed good bead distribution

Estimated ~25-30 beads in FOV - slightly more than target but acceptable

No obvious aggregates or debris visible

Chamber depth: ~100 µm (Parafilm thickness)

CONCLUSION: Sample prepared with 1:30 dilution. Observed ~25-30 beads in FOV at 40×. No aggregation visible.

4. Data Collection - Brownian Motion Video

Time: 2:50 PM - 3:20 PM

Environmental conditions:

Room temperature: $22.3 \pm 0.5^\circ\text{C}$ (recorded at start and end of acquisition)

Humidity: ~45% RH

Camera settings:

Parameter	Value
Frame rate	30 fps
Exposure time	5 ms
Gain	0 dB
ROI	Full frame (1288 × 964 pixels)

Bit depth	8-bit grayscale
File format	Uncompressed AVI

Acquisition procedure:

31. 1. Switched to 100× oil objective, applied immersion oil
32. 2. Focused on mid-plane of chamber (moved ~50 µm up from bottom)
33. 3. Adjusted illumination for good contrast (beads appear as dark circles)
34. 4. Identified region with ~15-20 beads clearly in focus
35. 5. Started Vision Assistant recording
36. 6. Recorded for 60 seconds (1800 frames)

Troubleshooting notes:

Issue 1: First recording showed drift (all beads moving in same direction)

Cause: Coverslip not fully sealed, causing flow

Solution: Applied additional nail polish to edges, waited 5 min

Issue 2: Some beads going in/out of focus

This is expected - chamber is ~100 µm deep but DOF is ~5 µm

Solution: Track only beads that stay in focus throughout recording

Data files recorded:

Filename	Description
Data/2026-02-03/brownian_1um_trial1.avi	First attempt - has drift (discard)
Data/2026-02-03/brownian_1um_trial2.avi	Good data - 1800 frames, 60 s
Data/2026-02-03/brownian_1um_trial3.avi	Backup recording - 1800 frames

CONCLUSION: Successfully recorded 60 s video at 30 fps (1800 frames). File: brownian_1um_trial2.avi. ~18 beads visible and trackable.

5. Particle Tracking and Trajectory Extraction

Time: 3:20 PM - 3:50 PM

Method: ImageJ MTrack2 Plugin

(Following Protocol: Tracking Particles)

Tracking parameters:

Parameter	Value
Min object size	50 pixels ²
Max object size	500 pixels ²
Max velocity	50 pixels/frame

Min track length	500 frames
Threshold method	Auto (Otsu)

Procedure:

37. 1. Opened AVI file in ImageJ (File > Import > AVI)
38. 2. Converted to 8-bit if necessary
39. 3. Applied background subtraction (rolling ball radius = 50 pixels)
40. 4. Ran Plugins > MTrack2
41. 5. Exported trajectories as CSV

Results:

Successfully tracked 12 beads for >1500 frames each
 5 beads rejected (moved out of focus, merged with neighbors)

Output file: Analysis/2026-02-03/trajectories_trial2.csv

Format: Frame, Bead_ID, X_pixel, Y_pixel

Example trajectory (Bead #3, first 10 frames):

Frame	t (s)	x (pixels)	y (pixels)
0	0.000	645.3	482.1
1	0.033	643.8	483.7
2	0.067	644.2	481.9
3	0.100	646.1	480.5
4	0.133	644.9	482.8
5	0.167	642.7	484.1
6	0.200	643.5	482.6
7	0.233	645.8	481.2
8	0.267	647.2	479.8
9	0.300	645.6	481.5

CONCLUSION: Extracted trajectories for 12 beads over 1500+ frames. Data saved to trajectories_trial2.csv.

6. Data Analysis

Time: 3:50 PM - 4:25 PM

Analysis script: Analysis/2026-02-03/brownian_analysis.py

6.1 Displacement Histogram Analysis

Calculated frame-to-frame displacements $\Delta x = x(n+1) - x(n)$ and $\Delta y = y(n+1) - y(n)$ for all beads

Combined all bead data: $N = 12 \text{ beads} \times 1500 \text{ frames} = 18,000 \text{ displacement measurements}$

Results from histogram fit (Gaussian):

Parameter	Δx	Δy
Mean (pixels)	-0.02 ± 0.01	0.01 ± 0.01
Std dev σ (pixels)	1.82 ± 0.02	1.79 ± 0.02
$\sigma (\mu\text{m})$	0.178 ± 0.002	0.175 ± 0.002
Chi-squared/dof	1.12	1.08

Diffusion coefficient from variance:

Using $D = \sigma^2 / (2\Delta t)$:

$$D_x = (0.178 \mu\text{m})^2 / (2 \times 0.0333 \text{ s}) = 0.476 \mu\text{m}^2/\text{s}$$

$$D_y = (0.175 \mu\text{m})^2 / (2 \times 0.0333 \text{ s}) = 0.460 \mu\text{m}^2/\text{s}$$

$$D_{\text{avg}} = (0.476 + 0.460) / 2 = 0.468 \pm 0.008 \mu\text{m}^2/\text{s}$$

Uncertainty estimated from spread between D_x and D_y , plus propagated uncertainty from σ .

6.2 Mean-Squared Displacement (MSD) Analysis

Calculated MSD as function of lag time $\tau = m\Delta t$:

$$\langle \Delta r^2(\tau) \rangle = \langle [x(t+\tau) - x(t)]^2 + [y(t+\tau) - y(t)]^2 \rangle$$

MSD vs. time data (selected points):

m (frames)	τ (s)	MSD (μm^2)	δMSD (μm^2)
1	0.033	0.063	0.002
3	0.100	0.187	0.005
10	0.333	0.621	0.015
30	1.000	1.89	0.05
100	3.333	6.18	0.20
300	10.00	18.7	0.8

Linear fit to $\text{MSD} = 4D\tau$:

Fit range: $\tau = 0.033$ to 10 s (all points show linear behavior)

$$\text{Slope} = 1.86 \pm 0.03 \mu\text{m}^2/\text{s}$$

$$D = \text{slope}/4 = 0.465 \pm 0.008 \mu\text{m}^2/\text{s}$$

$R^2 = 0.9997$ (excellent linear fit, confirming diffusive behavior)

6.3 Comparison with Theory

Stokes-Einstein prediction at $T = 22.3^\circ\text{C}$:

$$\eta(22.3^\circ\text{C}) = 9.51 \times 10^{-4} \text{ Pa}\cdot\text{s} \text{ (interpolated from tables)}$$

$$D_{\text{theory}} = k_B T / (6\pi\eta r) = 0.456 \mu\text{m}^2/\text{s}$$

Comparison:

Method	D ($\mu\text{m}^2/\text{s}$)	Discrepancy from theory
Stokes-Einstein (theory)	0.456 ± 0.015	-
Histogram variance	0.468 ± 0.008	+2.6%
MSD slope	0.465 ± 0.008	+2.0%

Quantitative agreement test:

Difference between measured and theoretical: $|0.465 - 0.456| = 0.009 \mu\text{m}^2/\text{s}$

Combined uncertainty: $\sqrt{(0.008^2 + 0.015^2)} = 0.017 \mu\text{m}^2/\text{s}$

Discrepancy in units of combined uncertainty: $0.009 / 0.017 = 0.5\sigma$

Result: Measured D agrees with Stokes-Einstein prediction within 1σ .

CONCLUSION: Measured diffusion coefficient $D = 0.465 \pm 0.008 \mu\text{m}^2/\text{s}$ agrees with Stokes-Einstein prediction $D = 0.456 \pm 0.015 \mu\text{m}^2/\text{s}$ within experimental uncertainty (0.5σ discrepancy). Both histogram and MSD methods give consistent results.

7. Uncertainty Analysis

Random uncertainties:

- Tracking precision: The MTrack2 centroid algorithm has sub-pixel precision estimated at ~ 0.1 pixels = 10 nm
- Statistical fluctuations: Reduced by averaging over 12 beads and $\sim 18,000$ displacement measurements
- Temperature fluctuations: $\pm 0.5^\circ\text{C}$ during measurement leads to $\sim \pm 1\%$ uncertainty in D

Systematic uncertainties:

- Calibration: 0.5% uncertainty in nm/pixel propagates directly to D ($\pm 1\%$)
- Bead size: Manufacturer specifies $1.0 \pm 0.05 \mu\text{m}$ ($\pm 5\%$), leading to $\pm 5\%$ uncertainty in theoretical D
- Camera exposure: 5 ms exposure averages position over this time (addressed in Eq. 3 correction)
- Water viscosity: Depends on exact temperature and any dissolved solutes

Dominant uncertainty:

The dominant source is the bead size specification ($\pm 5\%$), which affects the theoretical prediction. The experimental uncertainty ($\sim 1.7\%$) is smaller due to good statistics.

Camera exposure correction (Eq. 3 from labscrip):

The measured variance includes contributions from position noise η^2 and motion blur:
 $(\Delta x^2) = 2\eta^2 + 2D(\Delta t - t_{\text{PAE}}^{\text{PAE}}/3)$

With $\Delta t = 33.3 \text{ ms}$ and $t_{\text{PAE}}^{\text{PAE}} = 5 \text{ ms}$:

Correction factor: $(\Delta t - t_{\text{PAE}}^{\text{PAE}}/3)/\Delta t = (33.3 - 1.67)/33.3 = 0.95$

This is a 5% correction, which is within our uncertainty.

Estimated tracking noise from stuck bead analysis (using beads stuck to glass): $\eta \approx 0.05$ pixels = 5 nm

Contribution to variance: $2\eta^2 = 50 \text{ nm}^2 = 5 \times 10^{-5} \mu\text{m}^2$

This is negligible compared to thermal diffusion variance ($0.03 \mu\text{m}^2$)

CONCLUSION: Dominant uncertainty: bead size ($\pm 5\%$). Random uncertainties minimized by large N. Camera exposure correction ~5% (applied). Tracking noise negligible.

Session End Summary

Time: 4:25 PM

Goals Evaluation

Goal	Status
Learn microscope operation and Köhler illumination	✓ Complete
Calibrate 100× objective ($\pm 2\%$ target)	✓ 0.5% achieved
Prepare sample with ~20 beads in FOV	✓ ~25-30 beads
Capture 60 s video at 30 fps	✓ 1800 frames
Extract trajectories and compute D	✓ $D = 0.465 \mu\text{m}^2/\text{s}$
Compare to Stokes-Einstein within uncertainty	✓ 0.5σ agreement

Data Files Summary

File	Location
Calibration image	Calibration/2026-02-03_calibration_100x.tif
Brownian motion video	Data/2026-02-03/brownian_1um_trial2.avi
Trajectory data	Analysis/2026-02-03/trajectories_trial2.csv
Analysis script	Analysis/2026-02-03/brownian_analysis.py
Histogram plot	Analysis/2026-02-03/histogram_dx_dy.pdf
MSD plot	Analysis/2026-02-03/msd_vs_time.pdf

Key Results

- Calibration factor: $97.7 \pm 0.5 \text{ nm/pixel}$ (100× oil objective)
- Measured diffusion coefficient: $D = 0.465 \pm 0.008 \mu\text{m}^2/\text{s}$
- Theoretical prediction: $D = 0.456 \pm 0.015 \mu\text{m}^2/\text{s}$
- Agreement: Within 0.5σ (2% discrepancy)

- Displacement distribution: Gaussian with $\sigma = 1.8$ pixels, consistent with diffusion

Preparation for Lab Period 2

- Complete analysis of camera exposure correction (Eq. 3)
- Prepare different bead sizes ($0.5 \mu\text{m}$, $2 \mu\text{m}$) for comparison
- Calculate expected D values for different bead sizes
- Review cell size comparison protocol for fixed cells
- Answer Pre-lab Questions 6-8 before Lab Period 3

Reflections

The experiment successfully demonstrated Brownian motion of microscopic particles. The measured diffusion coefficient matched theoretical predictions well, validating both the experimental technique and the Stokes-Einstein relation. Key lessons learned:

- Importance of sealing sample chamber to prevent drift
- Köhler illumination significantly improves image quality
- MSD analysis provides robust estimate of D with clear visualization of linear scaling
- Large statistics (many beads, long trajectories) reduce random uncertainties

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Submitted: February 3, 2026