Advanced practical course
Experiment MMC

Biomolecular Motors:
From Cellular Function to Nanotechnology

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Heisenberg Professorship for BioNanoTools

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We will meet at 11.15 am at the reception of the MPI CBG (see map). Please get a visitor pass at the reception. A canteen/cafeteria for lunch is available. The whole lab course will be in English, this also includes your lab report.

Directions: Take tram no. 6 or no. 12 to the station Königsheimplatz. Cross the street and walk along the Schubertstrasse till you come to Pfotenhauerstrasse and turn left. Alternatively, take bus no. 74 or no. 62 to Johannstadt. Our institute is at Pfotenhauerstrasse 108. The building is green and blue.

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*If you like to present this experiment in the colloquium at the end of the semester, please contact your supervisor at least **one week** before your talk. Only then you can be provided with high resolution images or movies for the presentation. It is also recommended to present your slides to your supervisor before the seminar.*

1. Introduction

Cytoskeletal motor proteins, such as kinesins, dyneins and myosins, are essential in every cell. Their functions range from intracellular transport over the movement of muscles to chromosome segregation during cell division. In this practical we will focus on *kinesin-1*, a motor protein that moves along *microtubule* filaments. Kinesin-1 is involved in transporting membrane-bounded organelles - such as vesicles - over long distances through the cell (see Fig. 1).

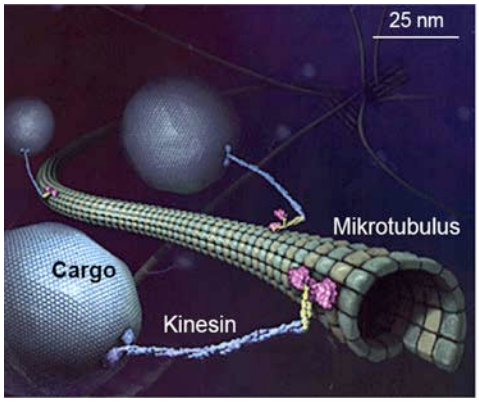


Figure 1: The motor protein kinesin-1 walks along microtubules, transporting vesicles from the cell center to the periphery.

The two most prominent setups for studying motor proteins outside cells are called motility assays and are depicted in Fig. 2. In *gliding assays*, the motors are immobilized on a surface and the filaments glide over the assembly (Fig. 2, left). In *stepping assays*, the filaments are laid out on the surface where they form tracks for the motors to move along (Fig. 2, right). In both assays, movement is observed under a light microscope using fluorescence markers or other high-contrast optical techniques.

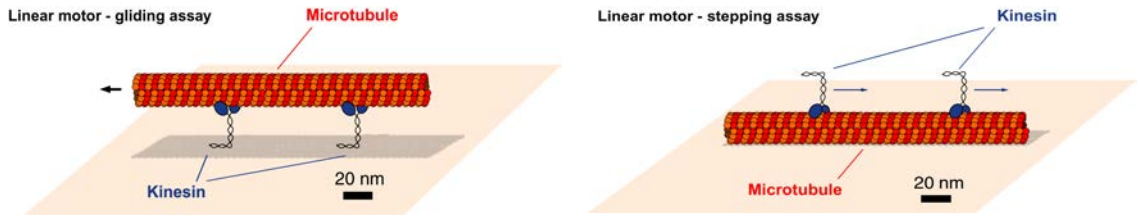


Figure 2: In-vitro motility assays. Left: Linear transport of filaments by surface bound motor molecules (gliding assay). Right: Linear movement of motor proteins along filaments (stepping assay).

Experiments to be performed: In this course you will perform a kinesin-1 stepping assay. Rhodamine-labeled, fluorescent microtubules (provided by the supervisors) will be bound to the glass surface using antibodies. You will use total internal reflection fluorescence (TIRF) microscopy to visualize single kinesin motors fused to green fluorescent proteins (GFP). Divided in two experimental groups you will measure the velocity and run length of single kinesin-1 motors walking on microtubules under two different conditions and decide whether they are significantly different. We expect you to interpret the results with respect to the mechanochemical cycle of kinesin-1 and/or basic biochemical processes. All images and videos will be acquired using *MetaMorph*, a software that controls the microscope and the camera system. Data processing will be performed directly after the experiments using two evaluation PCs in the lab and/or at home using custom written evaluation software (FIESTA) provided by the supervisors.

2. Material and Methods

2.1. Microtubules (MTs)

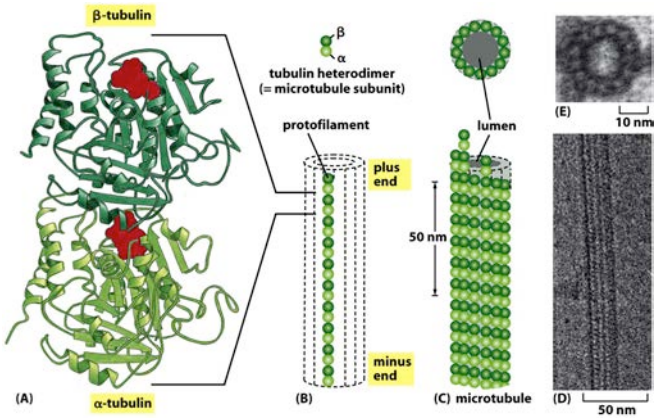


Figure 3: Molecular structure of a microtubule [1]

Microtubules are part of the cytoskeleton that forms a structural network in the cell. Microtubules serve as tracks for active intracellular transport and also play a crucial role in cell division. Their structure is shown in Figure 3: they are hollow polymer-cylinders having an outer diameter of 25 nm. Microtubules are made of α−/β−tubulin heterodimers held together by a non-covalent bond. These tubulin subunits (repeat length 8 nm) polymerize longitudinally with other tubulin subunits in a head to tail fashion to build up a protofilament. While, in living cells usually 13 protofilaments associate laterally to constitute the microtubule, the number of protofilaments of microtubules grown in vitro can vary. The uniform orientation of the tubulin subunits gives rise to a molecular polarity of the protofilaments, that results in a structural polarity of the microtubule (denoted as plus and minus end). This structural polarity allows for directed cellular transport along microtubules. This structural polarity is not to be confused with an electric dipole! The C-Terminus of tubulin comprises of the negatively charged amino acids glutamic acid and aspartic acid. This unstructured region is called E hook, faces the outside of the microtubule and facilitates interaction with positively charged proteins [Sirajuddin et al. 2014]. It serves also as a means of the cell to regulate microtubule protein interactions as the E-hook can be partially or fully cut by regulating enzymes.

Microtubules are highly dynamic filaments, which switch constantly between growing and shrinking phases (called ‘dynamic instability’). This dynamic behavior is driven by the reversible binding and hydrolysis of the nucleotide GTP by β−tubulin (GTP: guanosine triphosphate - an energy rich molecule; see Figure 3, red). (GTP also binds to α-tubulin, however it is irreversibly bound and never hydrolyzed.) Many proteins within the cell regulate growing and shrinking by increasing or decreasing the polymerization rates. Here, we will use Taxol-stabilized microtubules, which do not show ‘dynamic instability’.

Taxol is a microtubule-stabilizing agent. It binds to the β−tubulin subunit and presumably stabilizes the microtubule by straightening the protofilament (Caille et al., 2007). *In vivo*, the stabilization of microtubules prevents cell division, which is why Taxol is also used as an anti-cancer drug in chemotherapy.

MTs can grow up to lengths of several tens of micrometers. However, since they are very thin, their contrast in bright field microscopy is low. Labeling tubulin with a fluorescent marker (e.g. rhodamine) and using fluorescence microscopy resolves this problem.

2.2 Kinesin-1

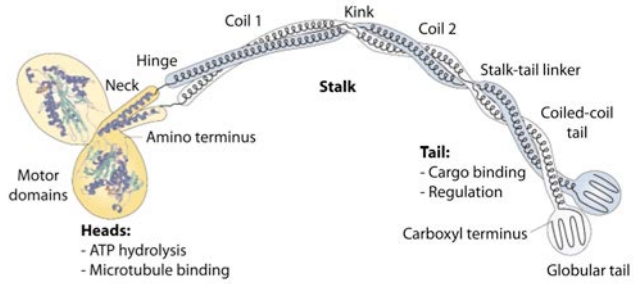


Figure 4: Kinesin-1 motor molecule [1]

Kinesin-1 (also called conventional kinesin) is a motor protein that, moving along MTs, transports cargo across cells. The molecule is a homodimer of two identical subunits forming a coiled-coil stalk (see Fig. 4) with a cargo-binding tail domain and two globular head domains. These head domains are also called *motor domains* because they are the force generating parts of the molecule. Kinesin-1 hydrolyzes ATP to move in discrete 8 nm steps and it is processive (it performs on average 100 steps before detaching from the MT). The chemical energy is provided by the hydrolysis of one ATP molecule per step. At saturated ATP concentration the kinesin motor reaches a speed of 0.8 µm/s (at room temperature) and exerts a force of up to 5 pN [2,6].

These qualities and recent findings employing single molecule techniques can be best explained by an asymmetric hand-over-hand mechanism [6]. Briefly this mechanism proposes a bipedal walk similar to our own. One head is attached to the MT at all times, while the free head moves around it to attach to the next tubulin dimer towards the plus end of the MT. This mechanism requires coordination between the heads to avoid detachment of both heads at the same time. Kinesin detaches from the MT when the gating mechanism fails and the first head detaches from the MT before the second head binds. This happens approximately in 1% of the steps.

The synchronization of the heads is thought to be facilitated by the neck linker region, which connects the motor domains with the coiled-coil part of the molecule.

Despite extensive research, some details of the mechanochemical cycle and its regulation are still under discussion.

2.3 Proteins, chemicals & other materials

BRB80 *(**B**rinkley **R**eassembly **B**uffer)*:

A buffer solution (80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA) of certain ionic strength to polymerize MTs (pH adjusted to 6.9 with KOH).

Taxol *(Paclitaxel)*: **WEAR GLOVES WHEN WORKING WITH TAXOL!**

Taxol stabilizes MTs and prevents depolymerization. It was discovered as anti-cancer drug, because it hinders the ‘dynamic instability‘ of MTs, which is necessary for cell division.

Anti-ß-tubulin antibody (anti-tubulin - SAP):

Anti-b-tubulin is a monoclonal antibody specific to b-tubulin, used to bind MTs to the surface of a cover slip.

F127: Pluronic F127 is a polymer that blocks hydrophobic surfaces against unspecific protein binding.

MgATP *(**Mag**nesium + **A**denosine **t**ri**p**hosphate)***:**

ATP provides the chemical energy source ("fuel") for the movement of kinesin. Magnesium is needed as a cofactor for ATP hydrolysis.
ATP hydrolysis: ATP $\hat{U}$ ADP + P_i + $101*10^{-21}$ J (at physiological conditions)
Note that the speed of ATP hydrolysis, as for any chemical reaction, is strongly dependent on temperature.

Casein: Casein is the principal protein fraction of milk. It blocks free surfaces against unspecific protein binding.

Antifade cocktail *(Oxygen scavenger system)***:**

D-glucose + Glucose oxidase + Catalase + BME or DTT. This cocktail works as an oxygen and free radical scavenger. Under strong optical excitation, the fluorophores form radicals, which react with oxygen and damage both fluorophores and proteins. The antifade cocktail reduces the amount of free oxygen reducing photobleaching and protein damage.

(1) $Glucose + O_2 + H_2O \xrightarrow{Glucose\ oxidase} Gluconate + H_2O_2$

(2) $2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$

2.4 Fluorescence Microscopy

Fluorescence is the dominant methodology used for labeling in biotechnology and biophysics. The detection of fluorescence is highly sensitive and has successfully replaced expensive and dangerous radioactive tracers that were used for biochemical measurements before. Imaging of fluorescent dyes in a cell can reveal localization of intracellular molecules down to the level of single-molecules (see Fig. 5).

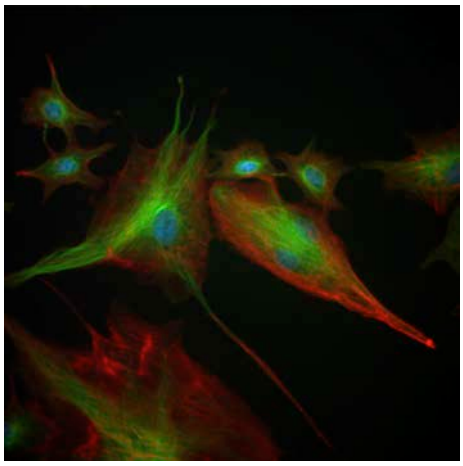


Figure 5: Image of fluorescently labeled MTs (green), actin filaments (red), and DNA (blue) in a cell.

Luminescence is the emission of light from any substance from electronically excited states. It is formally divided into fluorescence and phosphorescence – depending on the nature of the excited state (see Fig. 6).

Fluorescence: In excited singlet states, the electron in the excited orbital is paired (by opposite spin) to the second electron in the ground-state orbital. Consequently, return to the ground state is spin allowed and occurs rapidly by emission of a photon (emission rate ~10^8 s^{-1}).

Phosphorescence is the emission of light from excited triplet states, in which the electron in the excited orbital has the same spin orientation as the ground-state electron. Transitions to the ground state are forbidden and the emission rates are slow (10^3-10^8 s^{-1}). Following exposure to light, the phosphorescent substances glow for

several minutes (like “glow-in-the-dark” toys) while the excited molecules slowly return to the ground state. However, within this course we will focus on the rapid phenomenon of fluorescence.

Franck-Condon Principle:

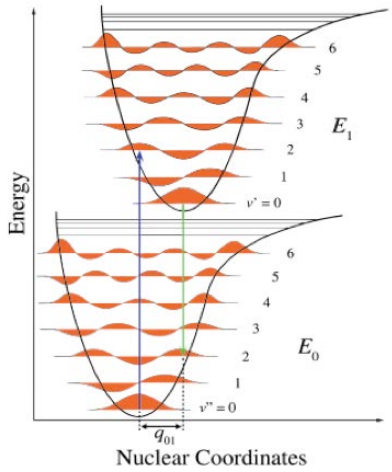


Fig. 6: Franck Condon Principle

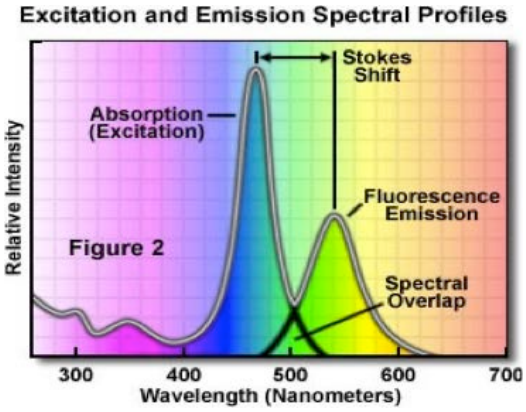


Fig.7: Typical Fluorescence Excitation and Emission Spectrum

Fluorescence of molecules displays a number of general characteristics, mostly originating from the Franck-Condon principle (see Fig. 6). In molecules, there are (3N-6) vibrational degrees of freedom all of which are rarely excited at room temperature. However, during electronic transitions caused, e.g. by light absorption, the final state is often vibrationally excited. The reason for this is, that nuclei have a high inertia compared to electrons which favors transitions with a minimal change in nuclear coordinates. The equilibrium nuclear coordinates change with the electronic state and with vibrational state, rendering it possible to keep the coordinates almost constant when the vibrational state is changed simultaneous with the electronic state. (see Figure 6). Vibrationally excited states are however quite unstable and relax fast to the ground state via transferring heat to the surrounding or reordering solvent molecules. The Franck-Condon principle leads to two effects:

1. The Stokes Shift

Emitted photons have lower energy (longer wavelength) than the excitation light as vibrational energy is redistributed to the surrounding.

2. Emission Spectra:

Fluorescence emission spectra are generally independent from the excitation wavelength as almost all transitions occur from the S_1 vibrational ground state into the same set of S_0 vibrationally excited states.

2.4.1 Fluorescent markers

Fluorescence probes are widely used in biology and thousands of these probes are known and can be divided in two classes – intrinsic and extrinsic.

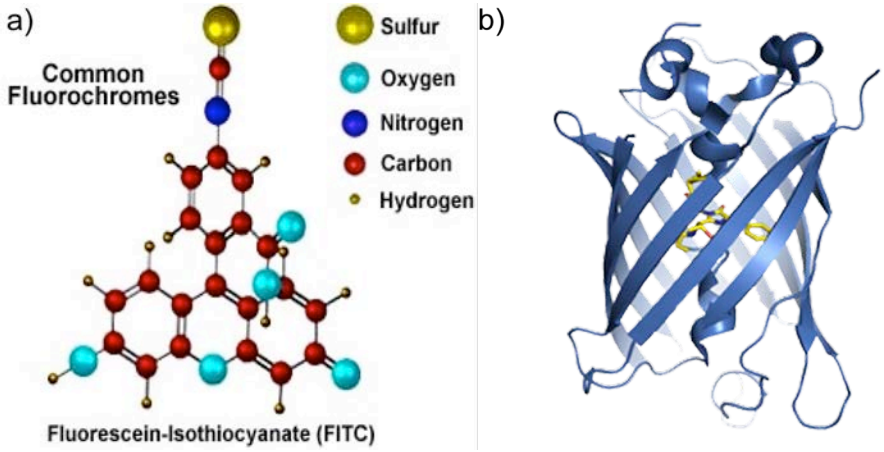


Figure 8: Structure of commonly used fluorescence probes. a) Fluorescein has several aromatic rings with delocalized π-electrons. b) GFP has a barrel-like structure with the chromophore positioned in the middle of the barrel [3].

Intrinsic fluorophores are those that occur naturally (e.g. aromatic amino acids or chlorophyll). Extrinsic probes are added to the sample to provide fluorescence when none exists, e.g. fluorescein (see Fig. 8a). They possess delocalized electrons in a conjugated p-electron system, where the first excited energy level of the molecule is relatively low and can be excited with the energy of visible light (in comparison to e.g. a hydrogen atom where the excitation energy is in the X-Ray range).

An important addition to the library of fluorescent probes was the green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria* (see Fig. 8b). It is possible to introduce the gene for GFP into cells, and to obtain proteins, which are expressed with attached GFP. Unfortunately all of these organic fluorophores display photobleaching (decay of emission intensity over time), especially in fluorescence microscopy with high illumination intensities.

2.4.2 Inverted fluorescence microscope

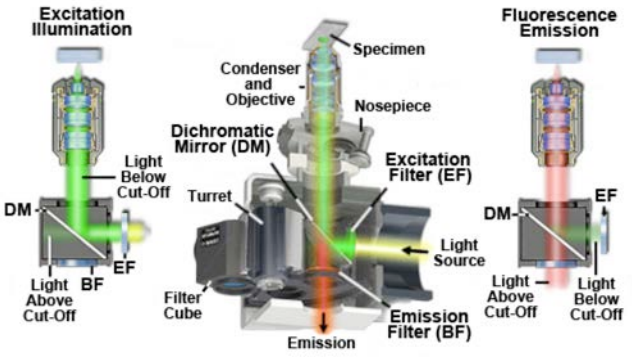


Figure 9: Basic setup of an inverted microscope [3]

A fluorescence microscope is an optical microscope, which is equipped with an excitation light source and different sets of mirrors and filters to create the optical paths for excitation and emission. A camera attached to the microscope allows for recording of digital images, which can be further processed by a computer. The term "*inverted*" refers to the position of the objective relative to the sample. Here, the objective is below the sample. The emission light is collected using the same objective but is separated from the excitation light by spectral filters.

2.4.3 Total Internal Reflection Fluorescence (TIRF) Microscopy

Total internal reflection occurs when a beam of light travels under an angle exceeding the critical angle Q_c from a medium of high refractive index towards a medium with low refractive index (e. g. from glass to water, see Fig. 10). Although the beam is completely reflected, an exponentially decaying evanescent field is generated which reaches typically 100 nm into the other medium. This is valuable in single molecule imaging because it provides a way to limit the volume where fluorophores are excited. Instead of a long, fully illuminated path through the sample, the effective illuminated volume is sub-wavelength in size and only fluorophores that are close to the surface will be detected. Thus, background fluorescence from the rest of the sample is largely suppressed.

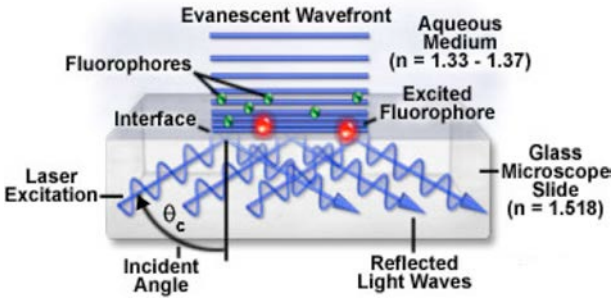


Figure 10: Schematic diagram of TIRF [3]

normal fluorescence TIRF

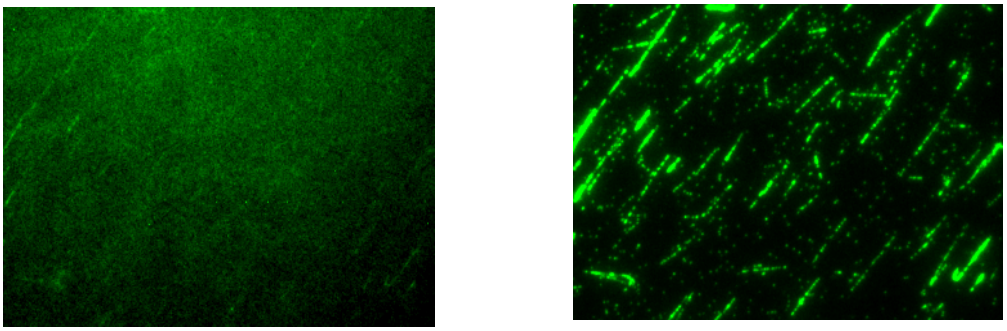


Figure 11: Fluorescence images of GFP-labeled kinesin motors on MTs and in solution in normal fluorescence and TIRF mode, where background fluorescence is strongly reduced.

2.5 Data Evaluation

The microscopy data will be evaluated using custom written software (FIESTA) that is available during the practical course, but can also be downloaded and used at home. Additional Information is available in appendix A2.

2.5.1 Statistical Significance and the Student's *T*-Test

To decide on the statistical significance of your data, you will be using the student's *t*-test. The student's *t*-test is a statistical method to help decide whether an experimental result is in accordance with a "null hypothesis". Commonly, the null hypothesis is that two results are equal (e.g. control and actual experiment). The students *t*-test takes into account the absolute value of the results, the standard deviation and the sample size. It then yields the probability to obtain the given result assuming the null hypothesis is true. Usually, the null hypothesis is rejected when the probability of obtaining the measured data is lower or equal to 0,05.

A special case of the student's t-test is the Welch Test, it is designed to work for unequal sample sizes and unequal standard deviations. Several types of T-Tests are implemented in spreadsheet software like Excel or OpenOffice as well as in

programming languages like Matlab or Python(scipy.stats).

3. Lab report

Every group will write up a summary of this lab course. It should contain a brief description of the experiment (*what* have you done and *why*), all relevant *graphs* and a *discussion* of the results with a *conclusion* that you draw from this lab course. For detailed questions, which have to be answered in the protocol, see the appendix section at the end of the document. The report should be structured in the following way (like any good protocol):

1 **Introduction:** Brief background, what is going to happen and why. Approximately one page.

2 **Experimental procedure: Do not** include the detailed protocols here. **Briefly** write in your own words what you did and why. Maximum half a page.

3 **Results:** Include four histograms of the velocities and run lengths for different buffer conditions. Estimate the characteristic parameters and compare distributions using statistical tests. Briefly describe how you performed your evaluation and what you observed.

4 **Discussion and Conclusions:** Discuss your data and draw your conclusions. In a good protocol, this part rounds up the story and refers to your introduction. (This is the part where we see whether you understood what you did!)

General hints for writing the protocol

- Every figure must be mentioned in the text (e.g. "see Fig. 1").
- Every figure must be understandable by itself. Use figure captions!
- Always put scale bars on your images!
- A histogram of a normal distribution should contain the mean value, standard deviation, and the number of data points N. It is nice, when the data is normalized.

4. Questions (be prepared to answer those at the beginning of the course)

1. Give a brief description of what constitutes a protein. What does primary, secondary and tertiary structure mean?
2. What are the main functions of microtubules in a cell?
3. Characterize the *kinesin-1* motor! (step size, energy consumption, walking model, function within the cell)
4. How does fluorescence work? Describe different fluorophores!
5. How does fluorescence microscopy work? What components are necessary?
6. What is the difference between normal fluorescence microscopy and TIRF microscopy? Draw a basic set up of an inverted fluorescence microscope!
7. Why should you always wear gloves when you work with Taxol?
8. How much BRB80 and 100 mM ATP solution do you have to mix to get 100 µl of 1 mM ATP solution? (1 mM = $1 \cdot 10^{-3}$ mol / liter)

5. Literature & Web links (for further information)

1. Molecular Biology of the Cell 4th Edition, 2002
   Bruce Alberts, Garland Science, London
   Everything you ever wanted to know about cell biology.
   Especially interesting: pp 907-923, 949-982, 68-91, 129-163.
2. Mechanics of Motor Proteins and the Cytoskeleton, 2001
   Jonathon Howard, Sinauer Associates, Sunderland
   Very good overview of the mechanisms controlling the cytoskeleton and motor proteins. Especially relevant: pp 151-165, 213-229.
3. Optical Microscopy Primers (from different companies)
   Nikon: http://www.microscopyu.com
   Olympus: http://www.olympusmicro.com
   Zeiss: http://zeiss-campus.magnet.fsu.edu
   Very good online introduction to microscopy.
4. Principles of Fluorescence Spectroscopy 3rd Edition, 2006
   Joseph R. Lakowicz, Springer, NewYork
   Very comprehensive book about fluorescence and its practical applications.
   Especially of interest for this practical are chapters 1 and 23.
5. HOWARD J, HUDSPETH AJ, VALE RD
   Movement of microtubules by single kinesin molecules.
   Nature 342:154-158 (1989)
   Motility assay.
6. ASBURY CL
   Kinesin: world's tiniest biped.
   Curr Opin Cell Biol, 17, (1), 89-97 (2005)
   Review about the kinesin stepping mechanism.
7. HIROKAWA N, TAKEMURA R
   Kinesin superfamily proteins and their various functions and dynamics. Exp Cell Res. 301, 50-59 (2004)
   Review about other kinesins and their functions.
8. VERBRUGGE S, VAN DEN WILDENBERG SMJL, PETERMAN EJG
   Novel Ways to Determine Kinesin-1's Run Length and Randomness Using Fluorescence Microscopy.
   Biophys. J. 97(8):2287-2294 (2009)
   Estimate the error of measured exponential distributions (e.g. run length)
9. SIRAJUDDIN, M., RICE, L.M., VALE, R.D.
   Regulation of microtubule motors by tubulin istoypes and post-translational modifications
   Nature Cell Biology 16, 335-344 (2014)

(PDF-files of References 5-8 can be found on our webpage: http://www.mpi-cbg.de/research/groups/diez/teaching.html)

Appendix. Experimental Details

Tip: Always take detailed notes of what you do and what you observe during all experiments. This is good practice for all scientific work and crucial to interpret your experimental results later on!

Kinesin-1 stepping assay

Both groups will immobilize rhodamine-labeled MTs on a glass surface using tubulin antibodies. They will be used as tracks for *kinesin-1* proteins. We use a kinesin-GFP fusion protein that allows us to localize the single molecules by their fluorescent signal when excited with a 488 nm laser line (protocols are provided on the day of your practical). You will acquire movie streams (continuous sequence of images with 100 ms exposure time) for two different buffer conditions.

The goal of the experiment is to quantify the mean velocity and the observed characteristic run length of at least 500 single kinesin-1 molecules for both buffers. A detailed step-by-step guide for collecting and evaluating the data is provided below. The main question to answer here is, how does the velocity and the interaction time depend on buffer conditions! Try to explain your findings.

Calculate the three characteristic parameters of kinesin-1 (velocity, interaction time, run length). Try to estimate the errors of the measured or calculated values. Discuss and compare your results with already existing publications (see chapter 5).

A.1 Stream acquisition using MetaMorph

A number of important steps have to be taken to ensure a proper data evaluation. This step-by-step guide and the highlighted questions should help to collect all necessary information.

1. When the flow cell is ready for imaging (see protocol), place the flow cell holder onto the microscope stage. Bring the oil objective into contact with the flow cell (the oil touches the bottom of the flow cell).
2. **What objective is used for imaging? What is the magnification? Which numerical aperture (NA) is used?**
3. Choose the <camera> button on the MetaMorph taskbar.
4. **What camera is used (working principle)? How much pixels does the camera have? What is the pixel size?**
5. The supervisor will choose the additional magnification (optovar).
6. **What additional magnification is used? (1.0x, 1.6x or 2.5x)**
7. **What does that mean for the pixel size in the "image plane"?**
8. Choose the TRITC filter on the taskbar, select Lamp illumination in acquisition dialog (drop-down menu bottom-left) and press <Show Live>.
9. A live image will be displayed. Focus to the MTs using the fine black focus knob at the microscope. You can move the objective closer to the sample by turning the right knob clockwise or the left knob counter-clockwise.

10. When the MTs are focused, press <Stop Live> to stop illumination. Press <Acquire> to take an image of the MTs.
11. Choose the GFP filter in the taskbar, select Laser illumination and press <Show Live>. The laser shutter is opened and a live image is displayed. Note, that the laser spot is visible at the ceiling. (Laser beam goes straight through objective). For laser safety reasons the supervisor will adjust the TIRF angle. Watch the fluorescence signal of the GFP-labeled motors on the live preview. ***Can you make out the transition to the TIRF mode?***
12. Again, choose the TRITC filter on the taskbar, select Lamp illumination and press <Show Live>. Move the sample to a new field of view, focus properly and take an image of the MTs.
13. Save this image! (The filename should contain important details: What is seen? Which filter is used? Which Magnification? Number of experiment? Excitation power? Optovar?)
14. Again, choose the GFP filter in the taskbar, select Laser illumination and press <Show Live>. **Do you see moving motors*?*** Press <Stop Live> and go to the <Acquire> menu and choose <Stream acquisition>. Fill the "number of frames" field with 1000. Make sure that the "<laser shutter>" is displayed in this submenu.
15. Press <Acquire> in the stream acquisition menu.
16. A stream with 1000 frames will be collected.
17. **What is the total time this stream will take? How does streaming actually work?**
18. Save this stream! (The filename should contain important details: What is seen? Which filter is used? Which Magnification? Number of experiment? Excitation power? Number of Frames? others)

Now, we can extract the data from the recorded streams.

A.2 Image processing with FIESTA and data evaluation

The experimental data will be extracted using FIESTA. There are three evaluation PCs available in the lab. Additionally you can download and use FIESTA at home. Data collection & evaluation will take about 1 to 2 hours.

Download: http://www.bcube-dresden.de/FIESTA

A.2.1. Data collection

During the experiment video streams (.stk files) of moving fluorescent motors were recorded. To extract the data, namely the velocity, out of the streams proceed as follows:

1. Open FIESTA
2. Load the stream (.stk file) via Data > Open stack
3. Enter pixel size in nanometer
4. Start evaluation via Statistics > Kymograph Evaluation
5. Click on Filament Image
6. Load the according filament image > displays microtubule image
7. Manually select microtubules with line tool (optional: adjust line width)
8. Click on Create: creates kymographs for all selected microtubules
9. A randomly selected section is shown (red lines indicate end of filament)
10. Find clear single molecules traces on the microtubule and select them by
    i) using the line tool (manually) or
    ii) by pressing the scroll wheel or middle button (automatic)
11. After you measured **every clear trace in this section**, click on Next
12. Repeat till you measured at least 200 molecules, then click on Export

Saved files include the filament image (with the selected microtubules) all kymograph images and the analyzed traces in MATLAB and ASCII format.

A.2.2 Data evaluation of velocity (at home)

1. Plot a histogram of the measured velocities for both conditions. The histograms should be compared visually (qualitative). Here, it is important to use the same scale, bin size and bin location for the two histograms comparing the same quantity. Choose a good bin size, every histogram should have 5-10 filled bins and the bin size should be recognized easily (e.g. 0.5 instead of 0.4736). Use a probability distribution (normalize "counts" by number of measurements).
2. **What is the underlying basic distribution? Is there a test to support your claim?** See statistical hypothesis test in A.2.4
3. Choose a reasonable way to calculate the mean velocity, the standard deviation, and the standard error of the mean velocity. Do not use the binned data from the histograms in order to avoid any influence from binning. Note how many events you have (total number of observed molecules).
4. **What measures could be taken in order to make the velocity measurement more accurate?**
5. How does the kymograph evaluation tool influence the statistical error? Perform test measurements to support your assumption.
6. **Compare the velocity of kinesin-1 under both conditions! What test can you use to quantitatively support your hypothesis?**

A.2.3 Data evaluation of run length (at home)

1. Plot a histogram of the measured run lengths for both conditions. The histograms should be compared visually (qualitative). Here, it is important to

use the same scale, bin size and bin location for the two histograms comparing the same quantity. Note down the bin size that you have used.

2. **What is the minimal reasonable bin size for your data? How many frames do you need to detect a "running" motor?**
3. **What follows for the first bin of your distribution? Do you have to treat these molecule traces differently?** See Figure A1.
4. **Assume an exponential distribution (include the equation of an exponential distribution)!** Define measurement limit x_0 (minimum value where distribution starts to show expected behavior). Disregard measurements below the defined limit x_0.
5. Calculate the cumulative probability density and fit a modified cdf to your data:

$$cdf(x) = 1 - e^{-k\cdot(x-x_0)}$$

6. Why do we prefer the cumulative distribution function (cdf) method to the probability density function (pdf) method?
7. Calculate the characteristic run length and the error of the runlength (from the cdf). Note how many events you have (total number of observed molecules).
8. **What measures could be taken in order to make the run length measurement more accurate?**
9. How does the kymograph evaluation tool influence the statistical error? Perform test measurements to support your assumption.
10. **Compare the run length of kinesin-1 under both conditions! What test can you use to quantitatively support your hypothesis?**

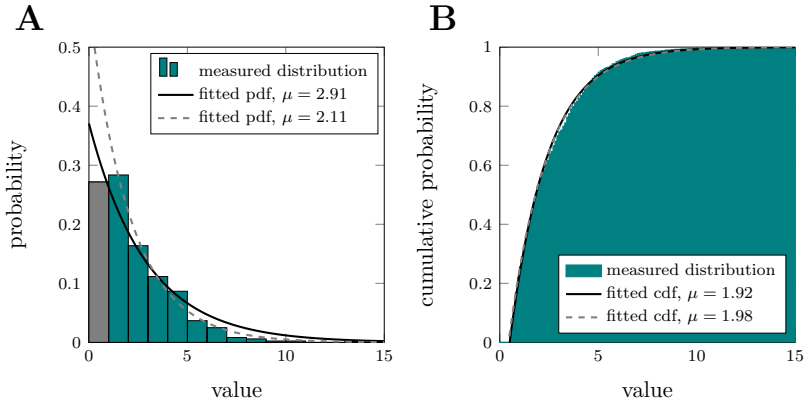


Figure A1: Simulated distribution with μ=2. (A) Probability distribution fitted by an exponential probability density function (pdf) to all bins (black solid line) versus all bins except first bin (gray dashed line). (B) Cumulative probability density fitted by a cumulative distribution function (cdf) with a constant offset x_0 (black solid line) or x_0 included as a fit parameter (gray dashed line).

Do not forget to explain how you actually measured the velocity and run length of the *kinesin-1* motors.

A.2.4 Statistics in data evaluation and hypothesis testing

Recommended literature and online tools for statistics and hypothesis testing:

Statistical Data Analysis, 1998
Glen Cowan, Oxford University Press, Oxford

Introduction to Statistics and Data Analysis for Physicists
G.Bohm & G. Zech, Verlag Deutsches Elektronen-Synchrotron, Hamburg
eBook: http://www-library.desy.de/elbook.html

Statistics at the Bench, A Step-By-Step Handbook for Biologists, 2009
M. Bremer & R. W. Doerge, Cold Spring Harbor Lab. Press, Cold Spring Harbor

Free Statistics Software, 2012
P. Wessa, Office for Research Development and Education
http://www.wessa.net/

Statistics to Use, 1996
T.W. Kirkman, College of Saint Benedict and Saint John's University
http://www.physics.csbsju.edu/stats

A.2.5. Discussion

What do the above observations with respect to velocity and run length mean for the transport mechanism of cargo by several *kinesin-1* molecules within the cell? Discuss the biological role of *kinesin-1* with respect to the observed behavior of the motor proteins (especially in the case of long range transport of vesicles, e.g. several cm to m).

A.2.6. Statistics in data evaluation and hypothesis testing

Recommended literature and online tools for statistics and hypothesis testing:

Statistical Data Analysis, 1998
Glen Cowan, Oxford University Press, Oxford

Introduction to Statistics and Data Analysis for Physicists
G.Bohm & G. Zech, Verlag Deutsches Elektronen-Synchrotron, Hamburg
eBook: http://www-library.desy.de/elbook.html

Statistics at the Bench, A Step-By-Step Handbook for Biologists, 2009
M. Bremer & R. W. Doerge, Cold Spring Harbor Lab. Press, Cold Spring Harbor

Free Statistics Software, 2012
P. Wessa, Office for Research Development and Education
http://www.wessa.net/

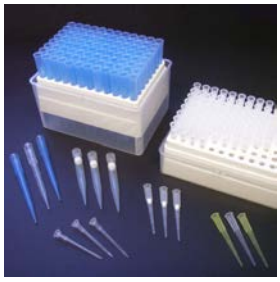
Statistics to Use, 1996
T.W. Kirkman, College of Saint Benedict and Saint John's University
http://www.physics.csbsju.edu/stats

A.3 Pipetting Made Easy

Mixing reagents has been important in chemistry for centuries with increasing importance in biology, biochemistry, biophysics, and nanotechnology over the last decades. Gilson™ adjustable micropipettes have been a great advance for measuring precise volumes by adjusting an air chamber through a strained spring. Although their handling seems quite easy, certain measures have to be taken to prevent damage to the mechanical tool.

Materials: one pipette holder; pipette set containing 2 µl, 10 µl, 100 µl, 200 µl and 1000 µl pipettes; pipette tips in sizes of 10 µl, 200 µl and 1000 µl; separate waste containers for pipette tips, 0.6 µl and 1.5 µl tubes, tube holder, stock solutions

Pipette set



Pipette tips

waste containers

tubes

Follow these steps carefully:

- **i.** Place the stock solution in the tube holder. Always start with the biggest volume and repeat the following steps until you reach the smallest volume. Choose destination tube with the desired final volume as close to the maximum volume of the tube as possible, but do not exceed the limit of the tube. **Always label the tubes to prevent a mix-up.**
- ii. Choose the appropriate pipette with the desired volume within the working range of the pipette. Type and range are indicated by the color cap (1). Selection of the pipette with the lowest maximum range above the desired volume will improve the measurement e.g. use the 100 µl pipette for 60 µl and not the 200 µl.
- **iii.** Hold the pipette with your dominating hand, the tip facing down, and adjust the volume by turning the volume knob (4) with your non-dominating hand. Turn clockwise to decrease the volume, counter-clockwise to increase the volume, until the pointer of the display window (5) reaches the desired

volume. **CAUTION: Do not adjust to values higher than the maximum working range.**

iv. Place the pipette back in the holder, the tip should always point downwards.
v. Open the tube containing the stock solution and place it back in the tube holder. Repeat the same with the destination tube.
vi. Pick up the pipette with your dominating hand, wrap the fingers around the finger rest (6) and place the thumb onto the plunger button (2). Thrust the tip connector (8) onto the opening of the appropriate pipette tip, which is kept in a holder. Use the 10 µl tips for the 2 µl and 10 µl pipettes; the 200 µl tips for the 20 µl, 100 µl and 200 µl pipettes; and the 1000 µl tips for the 1000 µl pipette. Check that the tip sticks tightly to the pipette by forcing it down onto the holder.
vii. Push down the plunger button (2) until your reach the first of two pressure points. Pick up the stock solution with your non-dominating hand and dip tip into the reagent. Make sure that the tip reaches the bottom of the tube to assure that no air bubbles reach the pipette tip.
viii. Slowly release the plunger button (2) upwards until the starting position is reached. Be aware of air bubbles which could corrupt the measurement.
ix. Place the stock solution in the holder, pick up the destination tube and transfer the tip into this tube. Press tip against the tube wall and push down the plunger button (2) to the second and last pressure point to release the solution from the tip. The tip should be directly in the solution if the tube already contains some liquid, which improves mixing of two or more reagents. **CAUTION: Do not tilt the pipette to prevent liquid flowing into the pipettes' mechanical system.**
x. Place the destination tube back in the holder and eject pipette tip into the waste container by pressing the ejector button (3). The ejection system (7) will forcefully release the tip; make sure to hold the tip into the waste container. **CAUTION: Always replace tip after every transfer to avoid contamination of your reagents.**

Repeat steps 2-11 until you mixed all of your reagents.