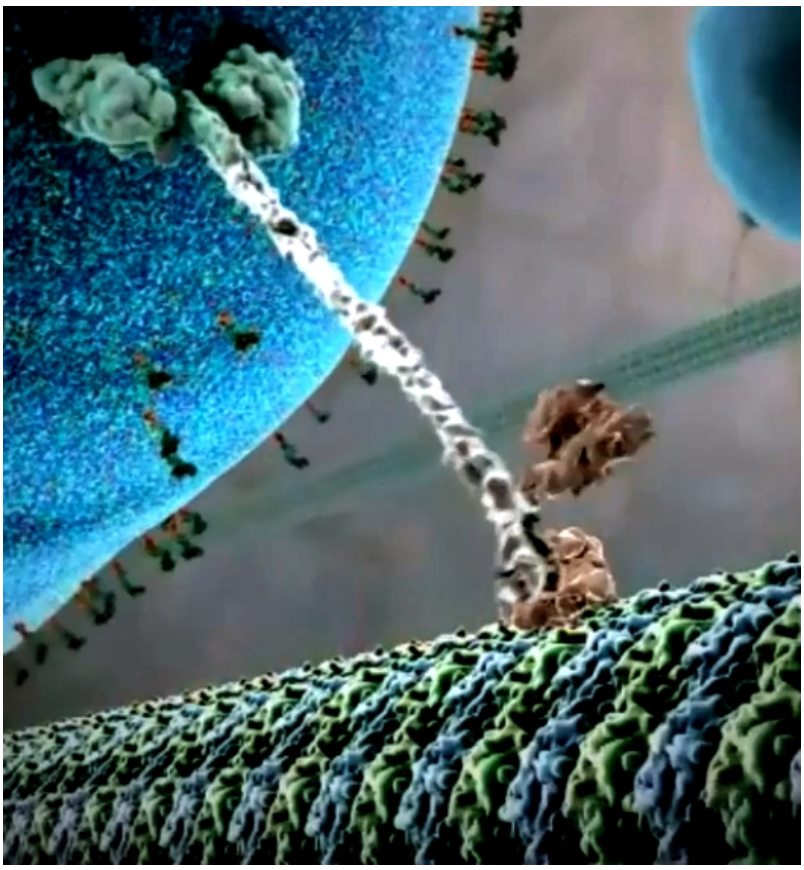

Biomolecular Motors: From Cellular Function to Nanotechnology (MMC)

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Title: Animation of a Kinesin motor protein carrying a vesicle. [2].

Contents

1 Introduction

In this experiment the influence of two different buffer solutions on movement behaviour of Kinesin-1 motors is to be determined using a stepping assay and TIRF microscopy.

The kinesin-1 motor protein represents one of the most important means of transport within a cell. By attaching its tail to a cargo and its heads to a microtubule, it can exert a force of about 5pN and move at a speed of about 8 nm/s. The locomotion results from a bipedal walk with a step size of about 8nm powered by the hydrolysis of ATP (one molecule per step).

Microtubule are part of the cytoskeleton and function as scaffolding as well as directed rails for intracellular transport. These straight tubular polymers consist of two different proteins, α - and β - tubulin that are stacked on each other forming long strands called protofilaments. The circular arrangement of these protofilaments makes the - normally dynamically unstable, e.g. constantly switching between growing or shrinking - microtubule. On average, they are 25μm long with an outer diameter of about 24nm.
In vitro one can combine motor proteins and microtubule in a two different settings: within a stepping array, the microtubule are mounted stationary while the porteins step along them. In a gliding array, the microtublue are pushed on top of the stationary proteins (see figure 1.1).

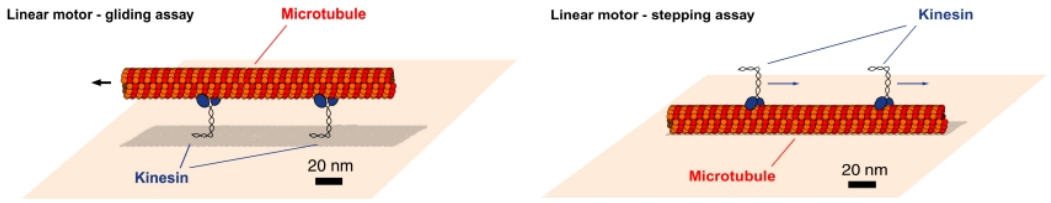

Figure 1.1: Motility assays. Left: fixed motor proteins push the microtubule (gliding assay). Right: motor proteins walk on fixed microtubule (stepping assay).[1]

To examine samples at this small scale, one can use flourescence microscopy. By adding flourophores to the specimen and exciting those in an inverted microscope, one can detect the emitted light and thus evaluate the inner specimen structure. For investigation of sample parts close to the surface (as in this experiment), a total internal reflection microscopy (TIRF) is an option. In this flourescence microscopy, the excitation light is reflected totally below the surface of the specimen, which causes an evanescent wave to occur at the surface (see figure 1.2). Thus, the excitation happens only to a depth of about 100nm and one gets a very distinct image of the structure therein, precluding any noise signals from deeper probe parts.

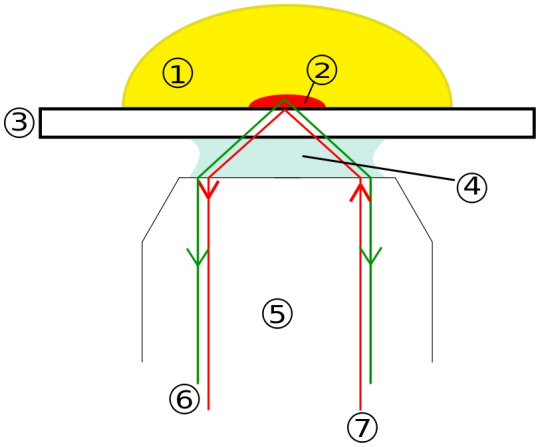

Figure 1.2: TIRF microscope scheme. 1: Specimen 2: Evanescent wave range 3: Cover slip 4: Immersion oil 5: Objective 6: Emission beam (signal) 7: Excitation beam. [3]

2 Experimental procedure

Movement behaviour of Kinesin-1 will be examined in two buffer solutions, one containing BRB80 (Brinkley Reassemblly Buffer), the other one BRB20Phos.

Three channels are prepared using parafilm on a microscope slide. To supply a certain pH value and environment for the microtubule, the channels are flushed with BRB80 (several times during the following procedure) and with Anti-Tubulin to enable attachment of them to the glass (these antibodies have a hyrophobic and a hydrophilic side and can thus stick to the glass as well as to the cover slip). F127 is added to hinder the kinesin from binding to the surface. Now, the microtubule are flushed in and are given time to attach. Finally, the kinesin solution with the buffer in question already added is flushed in and the specimen is ready to be examined.

For analysis we are using a TIRF microscope with a numerical aperture of 1.46, a magnification of 100x and a camera with a pixel size of 16x16μm^2. At first a picture is taken with normal light (see figure 2.1) to find micotubule, afterwards a short film of 1000 frames is taken with green laser light. The evaluation is done using the FIESTA program in Kymograph Evaluation mode. The program allows to generate time-translation images along previously marked microtubule. The movement of at least 200 (preferably 500) moving kinesin molecules is traced by hand, the program gives the distance and velocity data which are evaluated in Origin and Excel.

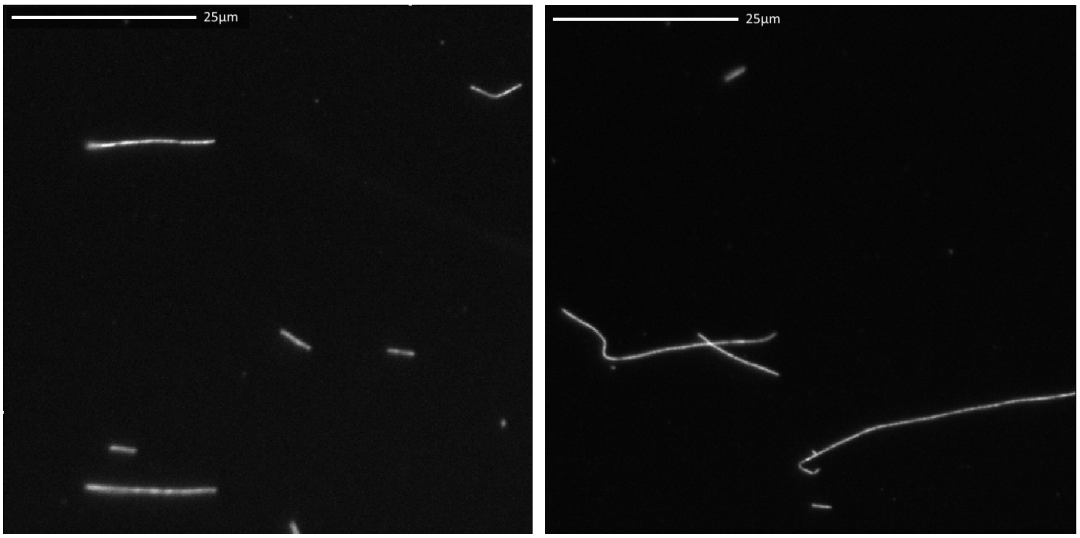

Figure 2.1: Microtubule within buffer 1 (BRB80, left) and buffer 2 (BRB20Phos, right).

3 Results

The number of acquired movement events varies between the two samples:

$$BRB80 : N_{80} = 115 \tag{3.1}$$

$$BRB20 : N_{20} = 497 \tag{3.2}$$

The temperature during the experiment was at 23°C.

3.1 Velocity

A histogram of the probabilites of the ocurring velocites is shown in figure 3.1 (bin size 0,2μm/s). The calculated mean velocities with the standard deviation as error values are

$$BRB80 : \bar{v}_{80} = 0.831 \pm 0.281 \mu m/s \tag{3.3}$$

$$BRB20 : \bar{v}_{20} = 0.795 \pm 0.314 \mu m/s \tag{3.4}$$

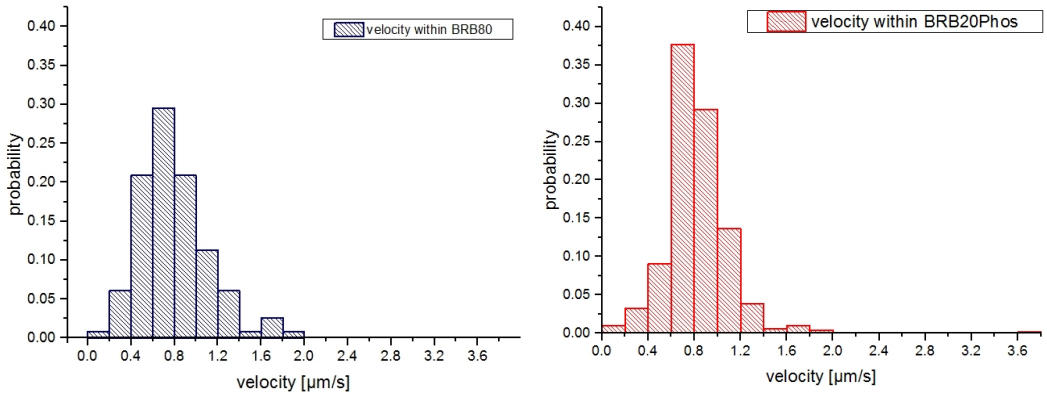

Figure 3.1: Velocity probabilites within buffer 1 (BRB80, left) and buffer 2 (BRB20Phos, right).

As one can see, the histograms form Gauss distributions that both peak at around the same mean value. This leads to the hypothesis that the two buffers might not have such a different effect on the motors in the end and that the velocities are statistically the same within both buffers. By executing a Welch test with Excel for the velocity data we acquire a p-value of

$$p = 0,255 \tag{3.5}$$

This is greater than 1% and thus, the hypthesis can not be neglected [5] and further investigation would be necessary to say if or with which probability it is true.
Since the TIRF microscopy itself is a very exact measurement (as seen in the pictures), the main error must stem from the FIESTA evaluation. Of course, the evaluation by hand is not as exact as an image processing program could be, so this might be an improvement. On the other hand, these errors should cancel out statistically (assuming there is no tendency for lines

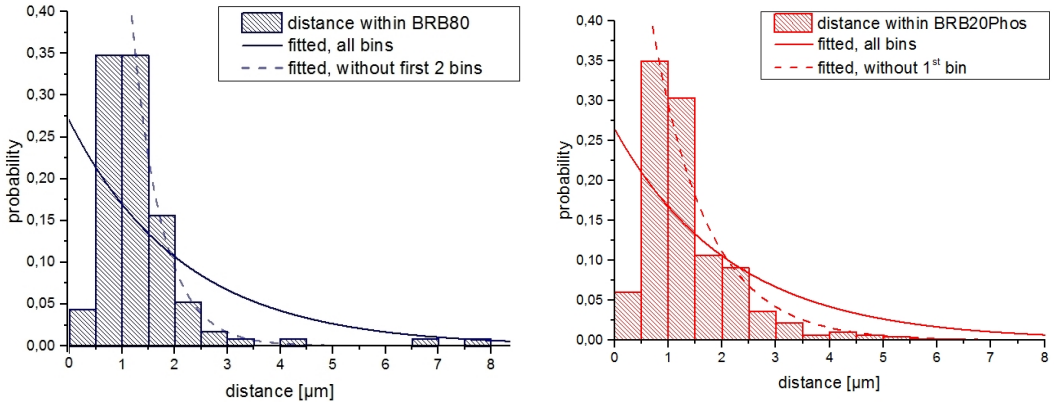

Figure 3.2: Run length probabilites within buffer 1 (BRB80, left) and buffer 2 (BRB20Phos, right).

of a specific length or slope). Hence, the main point is the loss of slow velocities due to the evaluation method: horizontal lines were not detected and assumed as non-moving. Very slowly moving proteins are thus being left out of the measurement.

3.2 Run length

A histogram of the acquired run lengths is shown in figure 3.2 (bin size 0,5μm). A minimal bin size would be the step size of the kinesin molecules (8nm) but for better visibility we used a larger one for the histograms. If we calculate the mean distances and the error by averaging or standard deviation of the raw data we get:

$$BRB80 : \bar{d}_{80} = 1.332 \pm 1\mu m \tag{3.6}$$

$$BRB20 : \bar{d}_{20} = 1.355 \pm 0.87\mu m \tag{3.7}$$

A Welch test as above gives us for the run lengths:

$$p = 0,823 \tag{3.8}$$

So again, we can not neglect the null hypothesis.

Now we want to fit an exponential function to the histograms. As explained before, the data for the shorter run length is not reliable. This is why we leave the first bin (in BRB20) or the first two (in BRB80) bins out of the fit. This yields these two funtions for the probabilites p of run length d:

$$BRB80 : p_{80} = 0.366 \cdot exp\left(-\frac{d - 0.782}{1.023}\right) \tag{3.9}$$

$$BRB20 : p_{20} = 0.345 \cdot exp\left(-\frac{d - 1.258}{0.568}\right) \tag{3.10}$$

The exact fit data for both curves (with all bins and with reduced bins) can be found in figure 3.3.

BRB20Phos

Modell	ExpDecay1		
Gleichung	y = y0 + A1*exp(-(x-x0)/t1)		
Chi-Quadr Reduziert	0,0068		
Kor. R-Quadrat	0,42561		
		Wert	Standardfehler
C	y0	0	0
	x0	1,69255	1,80745E7
	A1	0,12188	1,012E6
	t1	2,17675	0,94682

Modell	ExpDecay1		
Gleichung	y = y0 + A1*exp(-(x-x0)/t1)		
Chi-Quadr Reduziert	6,54677E-4		
Kor. R-Quadrat	0,94839		
		Wert	Standardfehler
C	y0	0	0
	x0	0,78168	3,43418E6
	A1	0,36605	1,22824E6
	t1	1,02347	0,11396

BRB80

all

Modell	ExpDecay1		
Gleichung	y = y0 + A1*exp(-(x-x0)/t1)		
Chi-Quadr Reduziert	0,00896		
Kor. R-Quadrat	0,35731		
		Wert	Standardfehler
A	y0	0	0
	x0	2,27827	2,54131E7
	A1	0,09376	1,10373E6
	t1	2,15872	1,06516

reduced

Modell	ExpDecay1		
Gleichung	y = y0 + A1*exp(-(x-x0)/t1)		
Chi-Quadr Reduziert	4,32657E-5		
Kor. R-Quadra	0,9954		
		Wert	Standardfehler
A	y0	0	0
	x0	1,2584	521094,23958
	A1	0,34533	316879,76863
	t1	0,56788	0,022

Figure 3.3: Data for all four fit curves in figure 3.2.

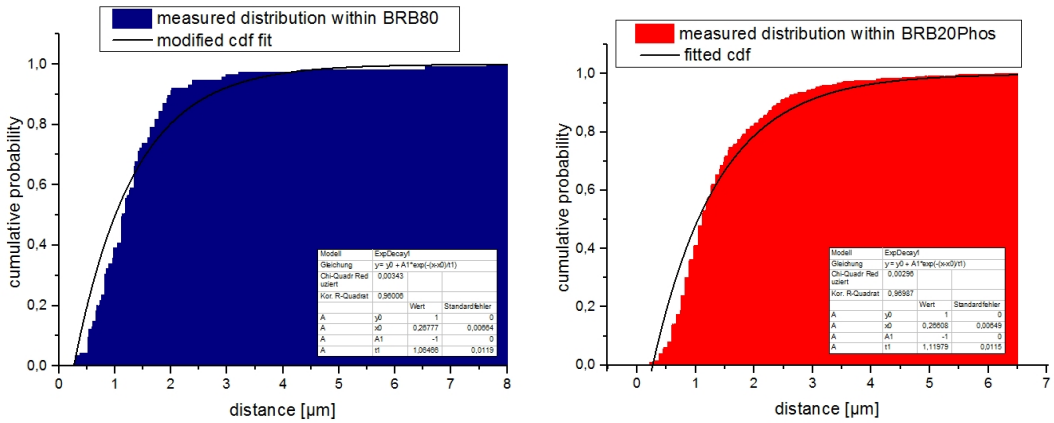

Figure 3.4: Cumulative probabilites and cdf function for run length within buffer 1 (BRB80, left) and buffer 2 (BRB20Phos, right).

The mean run length values obviously have an uncomfortably high error. We now try to determine more exact values by calculating them from the cumulative probability (see figure 3.4 - for higher accuracy we now use the minimal bin size of 8nm). One can clearly see that this has to be a better method in figure 3.4: the axis intercept within the cumulative probability diagram is not equal to zero and thus represents our real data a lot better than the non-cumulated probabilities.

We can approximate our cumulative probability distribution with a function of the form:

$$cdf = 1 - exp\left(-\frac{d - d_0}{t}\right) \tag{3.11}$$

For an exponential probability distribution of this form the median is [4]:

$$\bar{d} = ln2 \cdot t \tag{3.12}$$

So with our fitted cdf functions

$$BRB80 : cdf_{80} = 1 - exp\left(-\frac{d - 0.268}{1.065}\right) \tag{3.13}$$

$$BRB20 : cdf_{20} = 1 - exp\left(-\frac{d - 0.266}{1.120}\right) \tag{3.14}$$

we can calculate our optimised characteristic run lengths:

$$BRB80 : \bar{d}'_{80} = 0,738 \pm 0,008\mu m \tag{3.15}$$

$$BRB20 : \bar{d}'_{20} = 0,776 \pm 0,080\mu m \tag{3.16}$$

The error comes from Gauss' propagation of uncertainty ($\Delta\bar{d}' = ln2 \cdot \Delta t$), the values for Δ t can be found in figure 3.4.

3.3 Interaction time and needed frames

Using the acquired velocities and run lengths, we can now calculate the average interaction time $\bar{t}$ between a kinesin molecule and a microtubule. Again, the errors are calculated with Gauss.

$$\bar{t} = \frac{\bar{d}'}{\bar{v}} \tag{3.17}$$

$$BRB80 : \bar{t}_{80} = (0,928 \pm 0,600)s \tag{3.18}$$

$$BRB20 : \bar{t}_{20} = (0,933 \pm 0,330)s \tag{3.19}$$

These values seem indeed very similar and seem to support our hypthesis. The high errors can be explained by the small numbers off events we had available.

The number of frames needed to detect a moving motor depends on the Framerate F of the camera (assuming one can not see movement smaller than one step):

$$F = \frac{Numberoframes}{time} = \frac{F}{t} \tag{3.20}$$

with s = minimal step size = 8nm, v = velocity of the protein ≈ 0.8 μm/s:

$$v = \frac{s}{t} \tag{3.21}$$

It follows:

$$F = F \cdot \frac{s}{v} = 0.01F \tag{3.22}$$

4 Discussion and conclusion

Since all the data came from the same manual evaluation, it is not surprising that they all show the same behaviour: for both buffers the values are very close to each other, while the interaction time, velocity or run length is slightly larger for the kinesin molecules within BRB20. The t-tests we ran did not speak against the assumption that maybe both buffers have statistically the same effect on kinesin movement. On the other hand one has to admit that due to the small number of recorded events for the BRB80 solution, the data is not really comparable. Also it is important to say that the non-negation of the assumption does not mean it is true and a statistical similar effect does not mean the same thing happens on a molecular level.
The biggest source of error was certainly the manual evaluation, especially the disregard of slow movements and slow path lengths. One could try to include vertical lines in the Kymograph evaluation and to code an image processing program for the purpose of tracing the movements. We would assess this method of microscopy very yieldingly for this type of measurements.
For further research it would be good to gain a deeper insight on a molecular and chemical level in how the buffers are structured, what their chemical behaviour is like and how exactly they interact with the kinesin molecules and the microtubule.

Conclusion

In this experiment we succesfully examined the influence of two different buffer solutions on movement behaviour of kinesin-1 motors using a stepping assay and TIRF microscopy. In general the two buffers, containing BRB80 respectively BRB20Phos, did not show any difference in their impact on the kinesin movement. Statistical tests do not speak aggainst this hypothesis. Further investigation using more events and and an image processing program as well as a better chemial understanding would be necessary for more assumptions.

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