

TU DRESDEN

ADVANCED PRACTICAL COURSE

LAB REPORT

Biomolecular Motors

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1 Introduction

1.1 Kinesin-1: Cell's workhorses

All life forms in our universe are made of cells. To understand the mechanisms in and between those elementary building blocks of life is a real fundamental aim of biology, biochemistry and biophysics. But understanding these tiny but really complex structures also means understanding thousands of finely tuned mechanisms that are essential for survival of a cell. One necessary condition for most of these processes is a *directional* (not statistical!) motion of cell components. For example the segregation of chromosomes during cell division would not work with brownian motion alone. Fortunately there are so called *motor proteins* as kinesin, dynein and myosin that are responsible for these kinds of motion appearing in many transport-mechanisms.

In the following experiment we will investigate *kinesin-1*, a motor protein moving along *microtubule* filaments. For that purpose we will measure their velocity and the mean run length. But at first some basic knowledge.

Microtubules (MTs) are a component of the cytoskeleton that can be depicted with the "cell's highway" because they form basically the ground where the motorproteins can walk on. Their basic building blocks are the $\alpha - \beta$ -tubulin heterodimers that polymerise longitudinally in a periodic structure with a repeat-length of 8 nm. They form a structure of hollow-cylinders having an outer diameter of 25 nm. Because of the reversible binding and the hydrolysis of GTP(in figure 1 dyed red) by β -tubulin MTs show a highly dynamic growing behavior that is necessary to build those "roads" as flexible as possible. Later we have to surpass this effect. The directed motion on the MTs is enabled by the structural polarity of the tubulin-dimers. Figure 1 shows the strucuture of the MTs.[01]

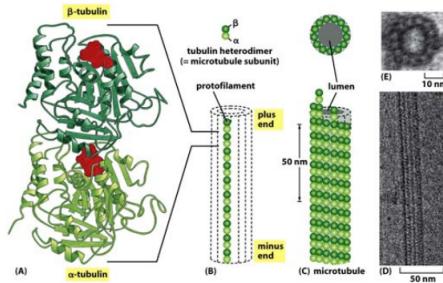


Figure 1: Structure of the microtubules[01]

Kinesin-1 is a motor-protein that moves along the MT transporting different types of cargo (e.g. vesicles, mitochondria, chromosomes) in the cells. It consists of two identical subunits that are winded into each other. For the motion from the minus-end to the plus-end of the MTs it uses the strucutural polarity of the tubulin-subunits binding its motor domain just to the β -tubulin parts. So it moves in discrete steps of $\Delta x = 8$ nm exerting a force of $F = 5$ pN to the MT-surface powered by the hydrolysis of an ATP-molecule during each step. The ATP-hydrolysis leads to an energy-emission of $E_{ATP} = 101 \cdot 10^{-21}$ J while the motorproteins do a work of $W_{MP} = F \cdot \Delta x = 4 \cdot 10^{-20}$ J. This means an efficiency of $\eta = W_{MP}/E_{ATP} = 39.60\%$.[01] The mechanochemical cycle of kinesin-1 is visualised in Figure 2:

1.2 Fluorescence Microscopy

In fluorescence microscopy the emission of light by a marked assay is used. More accurate, an electron in an excited singlet state within a molecule is paired by the contrary spin to the second

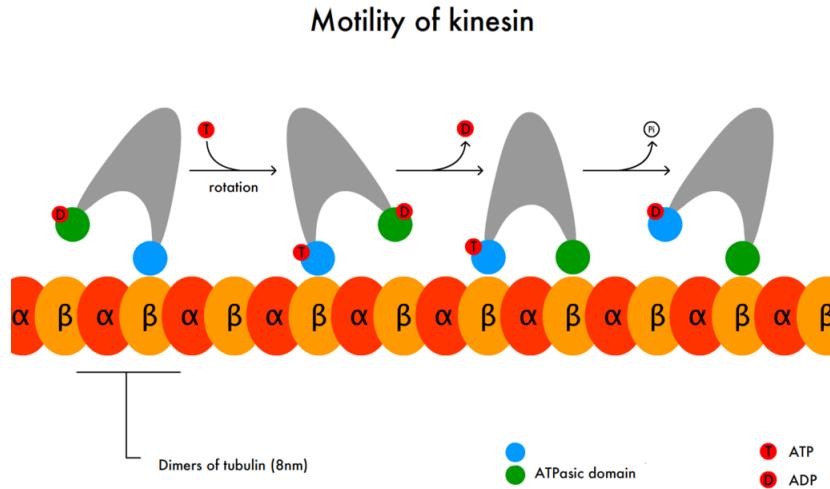


Figure 2: Mechanochemical cycle of Kinesin-1:
Absorbing of ATP, Emitting of ADP, ATP Hydrolysis[03]

electron in the ground-state orbital. The return to the ground state is allowed by spin (pauli principle). This appears rapidly with an emission rate of about 10^8 s^{-1} by the emission of a photon.

1.2.1 Fluorescent markers

There are two types of fluorescent markers: intrinsic and extrinsic. The intrinsic ones appear naturally in the sample (for example chlorophyll). Extrinsic markers are added to the probes, when fluorescence does not appear naturally (e.g. fluorescein). In a conjugated p-electron-system they have delocalized electrons. In this system, the first excitation energy of the molecule is comparatively low, so it can be stimulated by visible light. In this experiment, microtubules were marked with rhodamine and the motor proteins by green fluorescence protein (GFP).

1.2.2 Total internal reflection Fluorescence Microscopy

Total internal reflection fluorescence (TIRF) microscopy is used in this experiment. The base of this methodology is the total internal reflection. It can be detected when a beam of light travels from a medium with high refractive index to a medium with a low one under an angle bigger than the critical angle α_c . Even though the beam is totally reflected an exponentially decaying field called the *evanescent field* occurs. It reaches typically 100 nm into the medium with the lower refractive index. This distance is useful, because it naturally creates a way to limit the volume where fluorophores get excited. In this way the noise lowering the contrast between the signals and the background is suppressed.

2 Experimental procedure

2.1 Making of the Kinesin-1-stepping assay

We started the experiment with preparing the specimen that is visualised schematically in Figure 3. For that we got a hydrophobic object holder where the later mentioned antibodies and blocker-substances will bind to. Two flow channels were formed by placing 3 stripes made of paraffin on the slide that seal the channels under the coverslip after a short heating procedure. Then these

channels were flooded by several chemicals. At first we filled in the *BRB80* buffersolution. That is necessary to hold the pH-Value approximately constant. Then we put the *Anti- β -tubulin antibodies* in the channel that bind the microtubules to the surface of the object holder. The experiment of our group unfortunately failed probably because of using a too thin antibody-solution in this step. For hindering proteins other than the microtubules to bind to the surface of the slide we now wash the channel with some blocker-substances: *F127* and *Casein*. Because microtubules are highly dynamic filaments we have to stabilise them somehow. For this purpose we combine our BRB buffersolution with *taxol* that prevents the depolymerisation of tubulin. So finally we can put the rhodamine-labeled *microtubules* and the *kinesin-1 motorproteins* that are dyed with GFP in the channel. The kinesin-solution is enriched with *MgATP* that is the 'fuel' of the motorprotein's motion. Additionally a *glucose-based antifade-cocktail* is used to suppress the reaction of free-radicals created by strongly excited fluorophores with free-oxygen of about a factor 2. This is necessary to reduce the photobleaching and the damaging of the proteins.

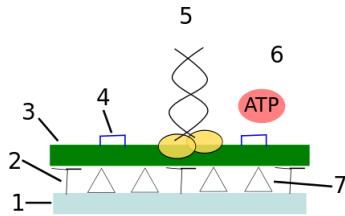


Figure 3: Schematic sketch of the stepping assay.
 1-Hydrophobic slide, 2-Anti- β -tubulin antibody,
 3-MT, 4-Taxol stabilisator, 5-motor-protein,
 6-ATP - 'fuel', 7-block substances (F127, Casein)

Now we are ready for taking the movies of our stepping-assay.

2.2 Stream acquisition

First the experimental supervisor placed the flow cell holder on the microscope stage. We used an oil objective which has to touch the bottom of the flow cell. The objective has a magnification of 100x and a numerical aperture of 1.46. After the assay was fixed we used a Software called MetaMorph. We used a digital Camera by Andor which took greyscaled pictures. Its resolution is 2.6 MPixel with 512x512 pixels. The size of one pixel ist 25600 nm^2 . According to the meta data of the movies and pictures the chosen additional magnification of the camera is 1.0x. This means, the pixel size is the original pixel size of the camera. If it had been 2.5x, we could have seen 2.5 times less, so a pixel would have been $A = 25600 \text{ nm}^2 / 2.5 = 10240 \text{ nm}^2$. So we took the TRITC filter and the lamp on the taskbar and watched the live images token by the camera. We searched and focused a cutout where we could see enough MTs. Unfortunately our assay showed that no MT's were fixed in it, so we could not take any pictures. Maybe this was caused by a too thin microtubule- or antibody-solution. We used the assay of our co-workers instead.

After we focused the MTs, we stopped live imaging and took pictures of the MTs luminescated by rhodamine. After that we take the GFP filter and choose the laser illumination. Then we choose again live imaging ("show live"). The TIRF angle will be adjusted. Again we choose the TRITC filter on the taskbar, the lamp illumination and take live imaging. We move to a new field of view, focus properly and take an image of the MTs such as in figure 4.

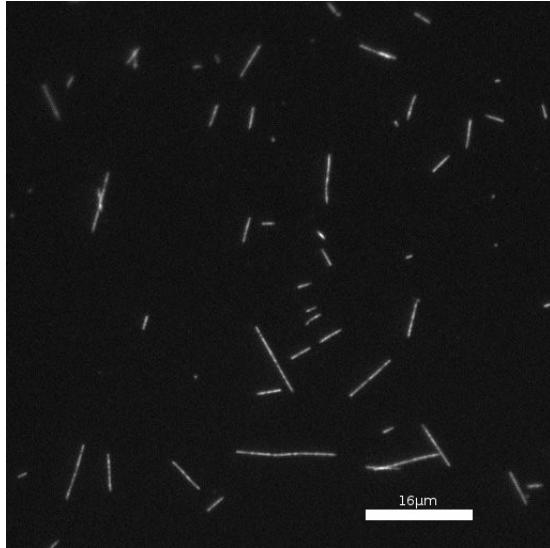


Figure 4: Example of an image, where we focused on the MTs

Then we save an image of this position. We now switched again to the GFP filter, selected the laser as light source and take live imaging. We can see moving motors. We stop live imaging and take a movie via the acquire button. The number of frames we took is 1000. For one frame the camera needs 150 ms, so at the end one we wait 150 s per video stream. We took 8 movies. When all streams are collected, one can do the data processing with FIESTA. There you can mark the possible trajectories of the motor proteins (as seen in figure 5) and the software will calculate pieces of the picture where the height is the time dimension and the width is the movement. If there is motion, one can mark up the lines and get the time which was needed for the marked distance (see figure 6).

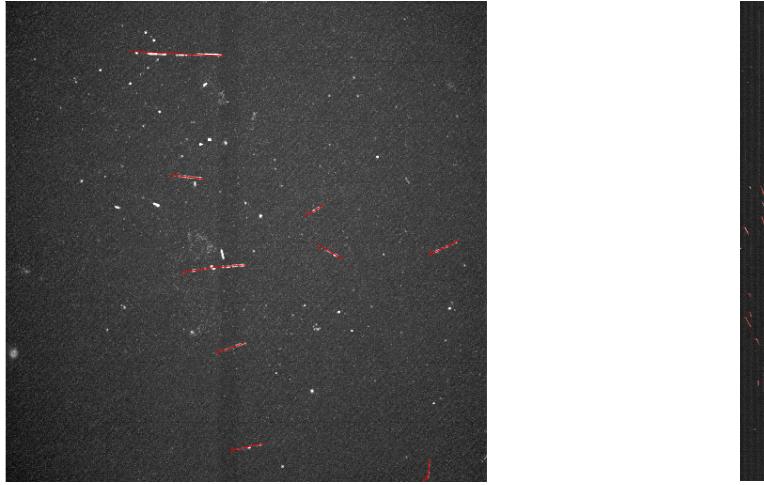


Figure 5: first step marking of the microtubules

Figure 6: marking of the trajectories of the motor proteins

3 Data Analysis

According to the data evaluation, we got $N = 204$ events. That means, we got 204 times a distance, a time and a velocity which are distributed.

3.1 Data evaluation of velocity

The mean of the velocity \bar{v} , its standard deviation $\sigma_{\bar{v}}$ and the standard error of the mean velocity $\Delta \bar{v}$ can be calculated by a data analysis tool. We used an Origin like software called qtiplot, which

calculates column statistics, which are not influenced by binning, because that are the measured raw data. These include the searched values. In general one can calculate these values as following:

$$\bar{v} = N^{-1} \sum_{i=1}^N v_i, \quad \sigma_v = \sqrt{\sum_{i=1}^N \frac{(v_i - \bar{v})^2}{N-1}}, \quad \Delta\bar{v} = \frac{\sigma_v}{\sqrt{N}}$$

So we get:

$$\bar{v} = (0.87 \pm 0.05) \mu\text{m/s}, \quad \sigma_{\bar{v}} = 0.72 \mu\text{m/s}$$

To get a better idea of the distribution of the values we created a histogram. Because we assume the velocity-values to be gaussian distributed we estimated a sensible bin-width w_{bin} with the help of *Scott's normal reference rule*[02]:

$$w_{bin} = \frac{3.5 \cdot \sigma_v}{N} \cong 0.4 \mu\text{m/s}$$

The fit function choosed to approximate the probability-density of the velocity v is given by:

$$pdf(v, A, \mu, w) = \frac{A}{w} \cdot \sqrt{\frac{2}{\pi}} \cdot e^{-2 \cdot \left(\frac{v-\mu}{w}\right)^2}$$

$$A = 0.40 \pm 0.01$$

$$\mu = (0.80 \pm 0.01) \mu\text{m/s}$$

$$w = (0.75 \pm 0.03) \mu\text{m/s}$$

For fitting the cumulative distribution of the gaussian we used a built-in function that approximates the exact form:

$$cdf(v, A, \mu, w) = \frac{A}{2} \cdot \left(1 + \operatorname{erf} \left(\frac{\sqrt{2} \cdot (v - \mu)}{w} \right) \right)$$

$$A = 0.991 \pm 0.002$$

$$\mu = (0.595 \pm 0.006) \mu\text{m/s}$$

$$w = (0.71 \pm 0.02) \mu\text{m/s}$$

Figure (7) shows the resulting histogram for the relative frequency and its cumulative distribution including all the fits

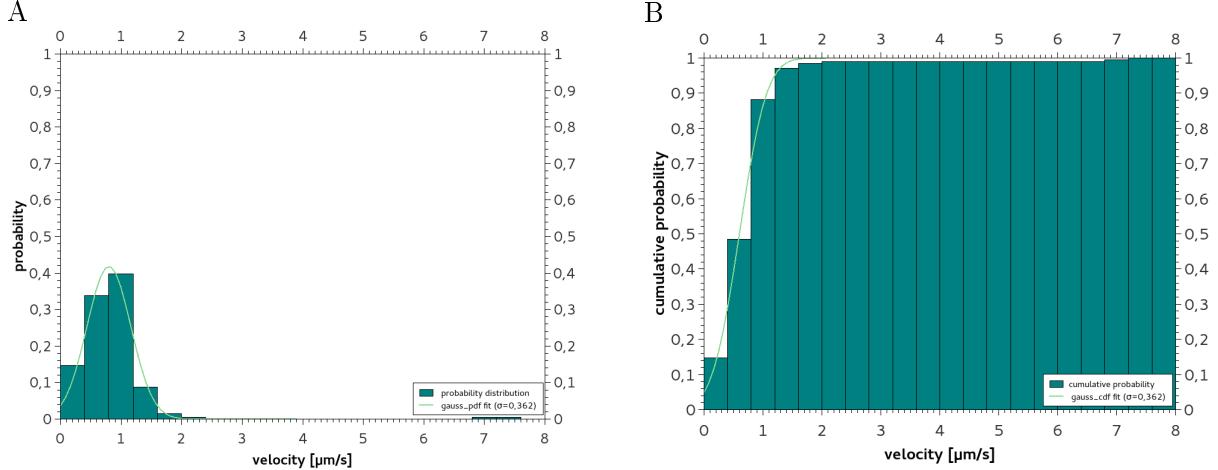


Figure 7: **A:** Histogram of the relative velocity distribution. Bin-size: $0.4 \mu\text{m/s}$ **B:** Histogram of the corresponding cumulative velocity distribution

To minimize the error one could easily take more measurements. According to the law of large numbers the measured mean velocity would converge to the expectation value. With an infinite number of measurements we would get the exact result. Also we could use software which marks the traces, which would create a unit law of marking. As a human being, one cannot see every trace and one can not mark every trace the way. This is caused by a diameter of the traces which is not infinite. The means, one could easily stretch the distance walked by the kinesin by marking from one edge to the diagonal opposite edge. An automation would avoid these errors.

3.2 Data evaluation of run length

Now we are going to figure out what distance D a motorprotein covers on a single microtubule before releasing itself. For that we also use the data acquired by the streamed films. One frame of the stream corresponds to 150 ms. With that and the determined velocity $\bar{v} = (0.87 \pm 0.05) \mu\text{m/s}$ we can calculate the minimal distance we can measure with our streaming-system (and also the minimal bin-size): $d_{min} = \bar{v} \cdot 1 \text{ FRAME} = 0.12 \mu\text{m} \cong 0.2 \mu\text{m}$. Therefore all of the measured distances which are below that value cannot be reasonable and they will be ignored in our statistics - anyway they will appear as first bin in the histogram for the sake of completeness. We rounded the value of d_{min} up because we want to choose a smooth bin-width. Finally we decided the bin width to be $w_{bin} = 0.4 \mu\text{m} \cong 2 \cdot d_{min}$. We estimated this using the **Square-root choice**[02] which says that the number of bins is $k = \lfloor \sqrt{N} \rfloor$ where $N = 204$ is the number of samples.

Considering these facts we got the following results. At first the mean distance and its statistical error:

$$\bar{D} = (1.1 \pm 0.1) \mu\text{m}$$

The figures (8) and (9) visualise the distribution of the measured distances:

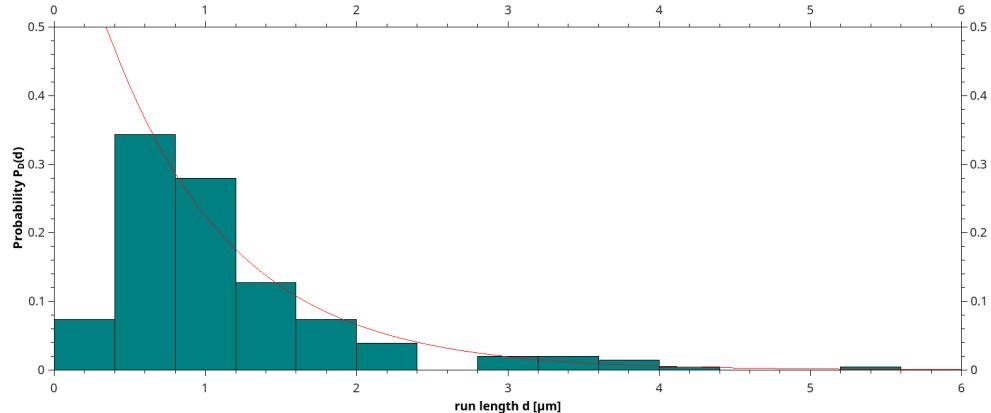


Figure 8: Probability distribution of the measured run length.

The very first bin is ignored from the exponential fitting.

Bin-size: $0.4 \mu\text{m}$

Because all invalid values fall into the first bin, it will be disregarded in the exponential fit of the probability function $P_D(d)$ and in the cumulative probability. We consider this fact by using an offset $d_0 = w_{bin} = 2 \cdot d_{min} = 0.4 \mu\text{m}$ in the exponential fit of the probability distribution. So the fit equation becomes:

$$P_D(d) = A \cdot e^{-\kappa \cdot (d-d_0)}$$

with the fit parameters:

$$A = 0.37 \pm 0.02$$

$$\kappa = (1.2 \pm 0.1) \mu\text{m}^{-1}$$

$$d_0 = 0.4 \mu\text{m} = \text{const.}$$

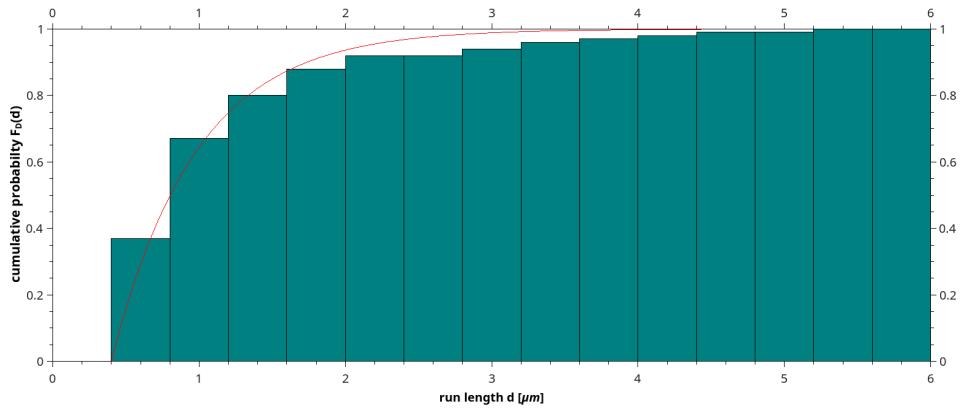


Figure 9: Cumulative probability disregarding the 'invalid' values with $d < d_0$

For the cumulative distribution function $F_D(d) = P_D(D \leq d)$ we use the fit equation:

$$F_D(d) = 1 - e^{-k \cdot (d - d_0)}$$

and extract the fit parameters:

$$k = (1.7 \pm 0.4) \text{ } \mu\text{m}^{-1}$$

$$d_0 = 0.4 \text{ } \mu\text{m} = \text{const.}$$

With that equation we can choose an arbitrary confidence level $F_D(d) \stackrel{!}{=} \pi(d) \in [0, 1]$ which gives the probability of the run length D to be less or equal than given d . If we invert this function we get the *characteristic run length* $d = \pi^{-1}(\pi(d))$:

$$\begin{aligned} d(\pi) &= d_0 + \ln((1 - \pi)^{-1/k}) \\ \Delta d(\pi) &= \left| \frac{\partial d}{\partial k} \Delta k \right| = \left| \frac{\Delta k}{k} \cdot (d_0 - d) \right| \end{aligned}$$

We calculate this length for some characteristic values of π that correspond to the confidence levels of the Gaussian normal distribution for finding the value of the random variable x in an intervall around the expected value: $x \in [-n\sigma + \mu, n\sigma + \mu]$, $n = 1, 2, 3$:

n	π	$d(\pi)$ [μm]	$\Delta d(\pi)$ [μm]
1	0.6827	1.1	0.2
2	0.9545	2.2	0.4
3	0.9973	3.9	0.8

We prefered the cdf-method to the pdf-method because in that way we may check our result for given π just by looking at the histogram without an additional integration. The deviation of the pdf-fit with respect to the histogram is also larger than in the cdf. We could minimise the error Δd easily by taking more samples. We also should have avoided motorprotein-trajectories that are too 'short' to minimise the amount of values within the first bin. Also the magnification of the camera could be choosed larger to follow the trajectories better in the software.

4 Discussion and conclusions

In this experiment we quantified the *velocity* v and the characteristic *run length* D (confidence level: 68.27 %) of *Kinesin-1 motor proteins* by doing a kinesin-1 stepping assay:

$$v = (0.87 \pm 0.05) \text{ } \mu\text{m/s}$$

$$D = (1.1 \pm 0.2) \text{ } \mu\text{m}$$

Those results fit well to the values given in literature: $v_{lit} = 0.8 \mu\text{m}/\text{s}$ [01] and $D_{lit} = (1.07 \pm 0.03) \mu\text{m}$.[04] So one single motorprotein does approximately 137 steps (8 nm each) before releasing itself. Those quantities give an impression of the meaning of Kinesin-1-motors in terms of long-distance-transport in cells: the run length of one single motorprotein is way too short to enable the motion over scales of centimeters or meters that are typical walking distances for the transport of e.g. neurofilaments in the axons of our nervous system. For that reason the molecular motors may transport cargo in a cooperative way: If we consider 8-10 kinesin-1 proteins they would be able to cover distances in macroscopic meter-scales.[05] Because the binding and releasing process to the MTs surface is statistically distributed several proteins have a lower probability of releasing all simultaneously, so the run length increases. The velocity of the proteins is influenced by the force imposed by the cargo: the higher the force the lower the velocity. If several motors pull the cargo then those forces distribute uniformly, so the run-velocity has to increase with the number of proteins. The investigation of these dependencies may be the object of further experiments or theoretical models.

References

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