Understanding *in vitro* microtubule degradation

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Abstract

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In this Ph.D. project, we aim to understand degradation of nanomachines by studying the mechanisms that lead to the in vitro degradation of molecular shuttles, which are nanoscale active systems composed of kinesin motor proteins and cytoskeletal filaments called microtubules. In addition, we aimed to improve learning outcomes by designing a hybrid college-level engineering course combining case-based and lecture-based teaching.

The creation of complex active nanosystems integrating cytoskeletal filaments propelled by surface-adhered motor proteins often relies on microtubules’ ability to glide for up to meter-long distances. Even though theoretical considerations support this ability, we show that microtubule detachment (either spontaneous or triggered by a microtubule crossing event) is a non-negligible phenomenon that has been overlooked until now. Furthermore, we show that under our conditions (100, 500, 1000 motors per µm² and 0.01 or 1 mM ATP), the average gliding distance before spontaneous detachment ranges from 0.3 mm to 8 mm and depends on the gliding velocity of the microtubules, the density of the kinesin motors on the glass surface, and time.

Wear, defined as the gradual removal of small amounts of material from moving parts of a machine, as well as breakage, defined as the rupture of a material, are two major causes of machine failure at the macroscale.1 Since these mechanisms have molecular origins, we expect them to
occur at the nanoscale as well. Here, we show that microtubules propelled by surface-adhered kinesin motors are subject to wear and breakage just like macroscale machines. Furthermore, the combined effect of wear, breakage and microtubule detachment from the surface of the flow cell permit to predict how molecular shuttles degrade \textit{in vitro}.

Taking a step back and looking at science in a broader sense, we can say that science does not only consist of acquiring knowledge, but also relies on one’s ability to transmit his/her knowledge. In this regard, one of the biggest challenges in education is to be efficient, that is to say to design a teaching method that would both maximize the student’s retention of information and prepare them to apply their knowledge to real-life situations. We considered this challenge as an integral part of this Ph.D. project, and we tackled it by designing a novel type of engineering course in which the students’ involvement in the learning process plays a central role. To do so, we combined, in a single engineering course, both of the approaches to learning that are used in Engineering education and in Business schools.

The final chapter of this manuscript summarizes the findings of the two projects presented here and discusses the future research that can be conducted on the basis of this thesis.
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The more one’s knowledge increases, the less zealous and intolerant one becomes, for intolerance stems from ignorance.

Ostad Elahi, Words of Truth
Chapter 1.

Background

The cytoskeleton of eukaryotic cells

The cytoskeleton of eukaryotic cells is a network made of dynamic protein filaments that cells use for intra- and extra-cellular processes such as: intra-cellular transport, mitosis, and interactions with the cell’s environment. The main filaments of the cytoskeleton are microfilaments, intermediate filaments, and microtubules. The filaments of interest in this thesis are microtubules.

Microtubules

Microtubules are hollow cylindrical structures assembled from thousands of α and β tubulin monomers. Each tubulin monomer is a protein that has a molecular weight of approximately 55 kDa and dimensions of approximately 4 nm x 4 nm x 4 nm. Each tubulin heterodimer (Figure 1, left) is constituted by one α and one β tubulin monomer that form αβ tubulin subunits. These subunits assemble “head-to-tail”, meaning that the β-tubulin of one heterodimer will bind to the α-tubulin of another heterodimer. This process will get repeated hundreds to thousands of times,
leading to the formation of long strands called protofilaments. Several protofilaments will then align in parallel and create a sheet of protofilaments, which slightly folds at each addition of a protofilament. Eventually, the sheet closes to create a microtubule. (Figure 1, right) A typical microtubule is composed of 13 protofilaments, but in vitro, the number of protofilaments can vary between 8 and 19 by changing buffer conditions during the growth phase of the microtubules, which modifies the diameter of the filament. Typical microtubules are 1-10 µm long, have an outer diameter of 25 nm and an inner diameter of 12 nm. In in vitro experiments, microtubules are often stabilized with paclitaxel, a small molecule which prevents microtubules from disassembling by non-covalently binding to tubulin, this stabilizing effect being reinforced by the kinesin-microtubule interactions.

Figure 1. Structure of a microtubule. Left – α- and β-tubulin monomers that constitute tubulin heterodimers. Center – a tubulin
heterodimer (top) and a representation of a protofilament formed by tubulin heterodimers assembled head-to-tail. Right – a microtubule formed by 13 protofilaments. Adapted from Alberts et al.6

When within cells, microtubules are dynamic proteins that are kept in dynamic equilibrium between assembly and disassembly, allowing for cell migration and spindle formation during mitosis.6,14 Typically, polymerization occurs more rapidly on what is known as the “plus end” of the microtubule, while depolymerization occurs more often on their “minus end”.15 Microtubules extend from microtubule-organizing centers (MTOC) out to the periphery of the cell,10 serving as tracks or “highways” on which cargo can be transported by molecular motor proteins.

**Molecular motor proteins**

Molecular motor proteins are biomolecules that can generate forces and/or movement. They can be divided into two categories:16 rotary motor proteins and linear or translational motor proteins. Rotary motors, such as the F0F1-ATP synthase, convert the energy stored in chemical gradients into kinetic energy, whereas linear motor proteins use the chemical energy stored in phosphate bonds to generate movement.

**Linear motor proteins and their movement**

The three main families of linear motor proteins are the myosin, the kinesin, and the dynein families (Figure 2). Linear motor proteins are usually associated with a cytoskeletal filament with which they bind in order to induce a movement. For instance, myosin attaches to actin filaments and creates the cross-bridge cycle that is at the origin of muscle contraction, and kinesin and dynein
can transport cargo along microtubules in a step-wise manner. This transport, however, takes place in opposite directions for the last two motor protein: inside a cell, kinesin motor proteins transport cargo along a microtubule from the MTOC towards the cell’s periphery, while dynein travels away from the periphery and towards the MTOC. This translates into kinesin motors travelling from the “minus end” of a microtubule to its “plus end” while a dynein motors travel from the “plus end” of a microtubule to its “minus end”. The sense of direction is conveyed through the recognition, by the kinesin heads, of the orientation of the asymmetric tubulin subunits of the microtubule.

Figure 2. Representation of the 3 main families of motor proteins. Adapted from Woelke et al.17
Kinesin

Kinesins are a family of linear motor proteins constituted of more than 30 proteins that share approximately 50% of their amino acid sequence with each other. In the rest of this thesis, the protein we will refer to as kinesin is kinesin-1. Kinesin-1 is a protein whose function is to transport vesicle within cells. It is a tetrameric protein made of two heavy (110-113 kDa) and two light (60-70 kDa) chains coiled around each other. The heavy chains constitute the heads, or motor domains of the kinesin, and the light chains constitute the kinesin’s tail. (Figure 3) The kinesin heads bind to the motor binding site of β-tubulin monomers, while its tail can bind to the cargo the molecule will be transporting.

![Figure 3. Representation of a kinesin-1 motor protein.](image)

We can observe the two heads of the kinesin and its coiled tail with light chains located at its distal end. Adapted from Pollard et al.18

Kinesin is a highly efficient processive protein that performs up to two hundred 8 nanometer steps along a microtubule before falling off,19 at a rate of about 100 steps per second. In vivo, the average kinesin speed is 1.5 µm/s,20 whereas in vitro, this speed rarely exceeds
0.8 µm/s. Kinesin’s walk along the microtubule is done in a hand-over-hand fashion, with a rotary stepping mechanism that generates torque and spins the cargo it is transporting. (Figure 4)

**Figure 4. Stepping cycle of a kinesin motor protein.** Kinesin heads hydrolyze ATP molecules to adenosine diphosphate (ADP) and phosphate ions. (A): Each kinesin head (blue) is bound to a tubulin heterodimer (green, β subunit; white, α subunit) along a microtubule protofilament. ATP binding to the leading head will initiate neck linker docking. (B): The “trailing” head of the motor is propelled forward toward the next tubulin binding site. (C): The new “leading” head attaches onto the binding site. The binding also accelerates ADP release, and during this time, the trailing head
hydrolyzes ATP to ADP-Pi. (D): After ADP dissociates from the kinesin “trailing” head, an ATP molecule binds to the “leading” head. The “trailing” head is in the process of being thrown forward.
Adapted from Vale et al.27

Molecular shuttles

In vitro, instead of the microtubules serving as immobile rails on which the kinesin motor proteins transport cargo, they are the moving elements of the system: kinesin genes are modified in order not to express their light chains, so that the kinesin tails can be physisorbed to a synthetic surface (such as glass).28 The motor is thus kept in place while the kinesin heads propel the microtubules using ATP as fuel.10 Such systems are called molecular shuttles. Molecular shuttles rely on active transport, which is an alternative to other biological transport mechanisms such as pressure driven flow or diffusion. Another name an assay in which microtubules are propelled forward by surface-bound kinesin motors is the “inverted motility assay”, the term inverted referring to the inverted geometry and distribution of mobile/immobile components of the system. (Figure 5)
Figure 5. Molecular shuttle. When the kinesin motor proteins are immobilized onto the coverslip, they will keep moving toward the plus (+) end of the microtubule. The sliding force is transmitted to the microtubule, which then moves in the direction of its minus (−) end. The casein proteins are added to the surface to let the kinesins anchor their tails and force them to “stand up” and expose their heads to the passing microtubules.

Molecular shuttles have been employed since 1999. They enable the controlled transport of molecules and other microscopic cargo in a synthetic environment. Biosensing, functionalizing microtubules with antibodies in order to capture molecules and viruses of interest, shuttle guiding in microfabricated channels, controlled activation of kinesin motors and options for using molecular shuttles for packaging and storage have been explored. A more recent advancement in the field is the use of molecular shuttles to travel through an extended network of junctions, which makes it possible to investigate applications of molecular shuttles such as motor protein-induced biocomputation.
In vivo and in vitro degradation of microtubules

The degradation of microtubules and other cytoskeletal filaments is often difficult to observe in vivo since cytoskeletal elements are constantly being assembled and disassembled according to the needs of the cell. For instance, in the case of microtubules, a number of proteins have evolved specifically to locally disassemble or disrupt supramolecular assemblies. For example, mitotic centromere-associated kinesins (MCAK) remove tubulin subunits from the ends of microtubules. As a result of this mechanism, cells are able to respond to a variety of stimuli and are also capable of moving. However, this process prevents the accumulation and subsequent observation of defective sites on the cytoskeletal elements in vivo.

In contrast, in vitro, the degradation of microtubules due to environmental stresses can often be clearly identified. Photodamage and oxidative damage of microtubules dramatically accelerate their disassembly, but unstabilized microtubules can respond to damage resulting from mechanical stress with self-healing if free tubulin is available. In addition to oxidative damage and photodamage, other factors also play an important role in the degradation of kinesin-powered molecular shuttles. As shown in Figure 6, the length distribution of microtubules bound to kinesin motors on a surface does not change over the period of an hour when the kinesin motors are inactivated by the molecule AMP-PNP (Figure 6, bottom row). In contrast, active transport of microtubules by ATP-fueled motors leads to the degradation of the molecular shuttles, with higher microtubule velocities being correlated to faster degradation of the nanosystem. (Figure 6, top 2 rows)
Figure 6. Evolution of microtubule population in the course of one hour, as a function of the ATP concentration in the flow cell. The ATP concentration determined the microtubules velocity in a molecular shuttle. The higher the ATP concentration, the faster the microtubule will be propelled forward. As of AMP-PNP, it is an ATP inhibitor. The first 2 rows show that microtubules’ velocity is correlated with their degradation, and the last row shows that is the action of the kinesin motors on the microtubules that cause their degradation. Scale bar: 20 µm. Adapted from Y. Jeune-Smith’s Ph.D. thesis.51
Wear at the nanoscale

The study previously conducted by Emmanuel Dumont highlighted one of the mechanisms that is at the origin of \textit{in vitro} microtubule degradation: nanoscale shrinking.\textsuperscript{3} Microtubules slowly shrink as they are propelled by surface-adhered kinesin motors. As illustrated in Figure 7, the shrinking rates depend on both the motor density and microtubule gliding velocity, the gliding velocity of microtubules being controllable by varying the ATP concentration in the solution. Furthermore, when looking at the dependence on the motor density, a transition from a low shrinking rate ($< 0.2$ nm/s) to a high shrinking rate ($>0.3$ nm/s) can be observed as the motor density increases from below to above 2,000 motors per square micrometre. This coincided with the mushroom-to-brush transition in the layer of surface-adhered kinesins, whose tail can be considered to be a freely-jointed chain.\textsuperscript{52}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{shrink_rates}
\caption{Shrinking of microtubules that are propelled by surface-adhered kinesins. \textit{Left} – Average shrinking rates and their standard errors as a function of kinesin density ($v = 199 \pm 3$ nm s$^{-1}$). The red triangle represents the average shrinking rate of stationary...}
\end{figure}
Rupture at the micro and nanoscale

In the past fifteen years, the study of protein degradation has led to rapid advances in the experimental\textsuperscript{53} and theoretical\textsuperscript{54} exploration of the effects of force on individual intermolecular bonds through the development of single-molecule techniques. These studies typically focus on rupture events\textsuperscript{55-57}, which are the result of a spontaneous escape over an energy barrier lowered by a constantly increasing force. However, these studies focus on the rupture of single molecular bonds, or of bonds between proteins. No study has looked into the rupture of supramolecular assemblies such as microtubules in \textit{in vitro} engineered systems such as molecular shuttles. This study is the goal of this project.

Engineering and business school education

Moving on to the second overarching goal of this thesis, one has to notice that engineering and business school courses both aim to teach students to solve problems, while the approaches they use to reach that objective are traditionally perceived as fundamentally different. Engineering courses provide the students with dense technical knowledge that helps them give a definitive answer to a problem, while business school classes focus more on collaborative learning by
confronting the students with real-world cases and by encouraging teamwork in order to find viable solutions to a question, the question not necessarily having a single black and white answer. We combined both of these approaches in a single course by designing the semester-long course “Fundamentals of Nanobiotechnology and Nanobioscience” directed towards senior undergraduate and graduate students. It incorporated key elements of the business school case study approach to learning, without altering the class time or the elements drawn from the traditional engineering education style. Our objectives for this project were both to create this course and to prove the effectiveness its hybrid format.
Chapter 2.

Microtubule detachment in gliding motility assays limits the performance of kinesin-driven molecular shuttles

Introduction

As described in Chapter 1, a molecular shuttle\textsuperscript{34,58-61} is a system capable of transporting molecules, vesicles, and other cargo from one location to another. Applications of such nanotransporters include biosensing,\textsuperscript{35} the study of collective motion\textsuperscript{62} and biocomputation\textsuperscript{46}. One of the most widely studied molecular shuttles consists of cargo-carrying microtubules being propelled on a surface by physisorbed kinesin motor proteins.\textsuperscript{40,63} Devices integrating such molecular shuttles rely on microtubules remaining attached to the kinesin-covered surface as they are going from point to point. For instance, the biocomputation device described by Nicolau et al.\textsuperscript{46} requires microtubules to glide over two hundred micrometers with a negligible probability of detaching. Plans for future generations of this device rely upon uninterrupted microtubule gliding over distances up to one meter.\textsuperscript{46}

A theoretical analysis by Klumpp and Lipowsky\textsuperscript{64} of the transport of nanoscale cargo by multiple kinesins along an immobilized microtubule can be applied to this inverse geometry (microtubules transported by immobilized kinesins). Similar to an earlier analysis by Hancock and
Howard, it predicts that the average distance a microtubule glides before detaching increases exponentially with the number of kinesin motors interacting with it, because individual motors have limited run lengths of the order of 1 μm. The model predictions are supported by experimental evidence for the transport of vesicles by 5-10 kinesin motors on microtubules over millimeter distances. However, for microtubules gliding on kinesin-coated surfaces, a much larger number of motors (10 – 1,000) can simultaneously interact with a microtubule. For typical microtubule lengths of 5 μm, a kinesin-microtubule interaction distance of 88 ± 1 nm, and kinesin densities between 20 μm⁻² and 1,000 μm⁻², the Klumpp and Lipowsky model predicts gliding distances between 0.2 m and 10¹⁷ m (10 light-years). For this reason, microtubule detachment during gliding is not expected to occur.

Experimentally, detachment of bundles of microtubules has been observed in the presence of depleting agents such as glycerol or methylcellulose, but single microtubule detachments in the absence of all depleting agent has not been investigated yet. We simulated the experimentally observed gradual depletion of gliding microtubules on the surface of the flow cell, modelling the evolution of microtubule density and length taking into account microtubule shrinking and microtubule breaking at rates matching the frequency of encountering a defective kinesin. Those simulations failed to match the experimental data until we included a detachment rate of gliding microtubules into them (Figure 20). Here, we experimentally measure the frequency of detachment of gliding microtubules and find that the average gliding distance is only on the order of 3 to 55 mm depending on motor density and gliding velocity.
Theoretical predictions

Spontaneous detachment of microtubules at a high rate have been previously observed by Howard et al. in experiments using very low densities of kinesin and short microtubules, where it is highly likely that the microtubule will be unable to reach another kinesin before it unbinds from the last kinesin holding it. Because the kinesins are randomly distributed on the surface, the number of motors covered by a microtubule is Poisson-distributed. Therefore, as derived by Howard et al., the probability $P$ for a microtubule not to find another kinesin motor in its path before it unbinds is given by:

$$P = e^{-w\sigma L} \quad (2.1)$$

where $L$ is the length of the microtubule, $w$ is the kinesin-microtubule interaction distance, and $\sigma$ is the density of kinesin on the surface. This width was calculated as the output from a computational model of kinesin head diffusion and binding to the microtubule by Palacci et al. and was found to be equal to $w = 88 \pm 1$ nm. The probability $p$ for a microtubule not to detach from the surface after gliding a distance $d$ is the cumulative probability of not detaching after each kinesin it encounters, which is given by:

$$p(d) = (1 - P)^N \quad (2.2)$$

where $1-P$ is the probability for the microtubule to find a kinesin in its path and $N$ is the number of kinesins encountered on its path. However, with the notations given above, we can write $N$ as $w\sigma d$. In other words, we can rewrite equation (2.2) as:

$$p(d) = (1 - P)^{w\sigma d} \quad (2.3)$$

We can rewrite equation (2.3) as the exponential function:

$$p(d) = e^{\ln((1-P)^{w\sigma d})} = e^{-\ln(1-P)^{w\sigma d}} \quad (2.4)$$

Thus, the expected average gliding distance $\langle d_H \rangle$ is:
The theory by Klumpp and Lipowsky takes a complementary perspective by modeling a fixed number of kinesin motors transporting a particle along a microtubule and accounting for the limited and force-dependent run length of the individual kinesin motors. Their model yields an estimate for the average travel distance of \(5^{(N-1)}/N \ \mu m\), where \(n\) is the number of motors. The full derivation of this formula is provided in their publication.\(^{64}\) This means that 10 motors can translate a particle approximately 1 meter without detaching. Applied to our situation where a certain number of kinesins attached to a surface (rather than the particle) interact with one microtubule, the number of motors \(n\) is given by \(Lw\sigma\) and the average transport distance is:

\[
\langle d_{KL} \rangle = \frac{5^{Lw\sigma-1}}{Lw\sigma} \times 1 \ \mu m \quad (2.4)
\]

The predictions of both theories for the average length of detaching microtubules in each of our experiments are shown in Table 1.

Ideally, a complete theory aiming to describe the detachment of microtubules while gliding on kinesin-coated surfaces would simultaneously account for the random distribution of motors and their limited run length. The theory would likely yield lower estimates than the two described approaches, but it would still predict an exponential dependence of the gliding distance on motor density and microtubule length. Neither the order of magnitude nor the functional dependence of the gliding distance matches the experimental measurements described in the following section. Indeed, as shown in that table, in our experimental condition with the lowest kinesin density (100 kinesins \(\mu m^{-2}\)), the microtubules should glide for 10 km before detaching, but we can still observe several detachments.
Table 1. Theoretically expected gliding distances of microtubules before detachment. In this table, $\langle d_H \rangle$ represents the expected average gliding distance, and $\langle d_{KL} \rangle$ represents the predicted average gliding distance according to the model developed by Klump and Lipowsky. The ATP concentration affects the velocity of the microtubules, thus influencing their shrinking and breaking rates. As a result, the average length of the microtubules (used as the “L” value in the distance calculations) is affected by it.

<table>
<thead>
<tr>
<th>Kinesin density ($\mu$m$^{-2}$)</th>
<th>ATP concentration (mM)</th>
<th>$\langle d_H \rangle$ (mm)</th>
<th>$\langle d_{KL} \rangle$ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>1</td>
<td>$1\times10^{60}$</td>
<td>$5\times10^{98}$</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>$3\times10^{198}$</td>
<td>$2\times10^{320}$</td>
</tr>
<tr>
<td>500</td>
<td>1</td>
<td>$1\times10^{71}$</td>
<td>$1\times10^{113}$</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>$8\times10^{109}$</td>
<td>$2\times10^{178}$</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td>$1\times10^{7}$</td>
<td>$8\times10^{10}$</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>$6\times10^{6}$</td>
<td>$2\times10^{13}$</td>
</tr>
</tbody>
</table>

Materials and Methods

Microtubules

Microtubules were polymerized from a 20 μg aliquot of rhodamine-labelled, lyophilized tubulin (Cytoskeleton Inc., TL670M) with 6.25 μL polymerization buffer. The polymerization buffer consisted of BRB80 buffer, with 4 mM magnesium chloride (MgCl$_2$), 1 mM GTP and 5% dimethyl sulfoxide. BRB80 buffer is composed of 80 mM piperazine-N,N′-bis(2-ethanesulphonic acid), 1 mM MgCl$_2$ and 1 mM ethylene glycol tetraacetic acid (EGTA), adjusted to pH of 6.89 with potassium hydroxide (KOH). The resulting solution was then incubated on ice for 5 minutes.
before being transferred to a 37 °C water bath for 30 minutes. The microtubules were then diluted a hundred-fold into BRB80 buffer and stabilized with 10 μM paclitaxel.

**Kinesin motors**

Kinesin from wild-type, full-length Drosophila was expressed by the team of G. Bachand at the Center for Integrated Nanotechnologies (Sandia National Laboratory) in *Escherichia coli* and purified using a Ni-NTA column. The kinesin was then nitrogen frozen in a buffer consisting of 40 mM imidazole, 300 mM NaCl, 0.76 g/L EGTA, 37.2 mg/L EDTA, 50 g/L sucrose, 0.2 mM TCEP, 50 μM Mg-ATP, the buffer being at pH 7. As measured from absorbance at 280 nm the concentration of the kinesin is 3.16 mg/mL. The kinesin density was computed from landing rate experiments (see below). The undiluted bulk solution of kinesin would result in our flow cells with a surface density of 11,000 ± 2,000 μm⁻².

**Flow cells**

Flow cells were assembled from a longer coverslip (60 mm x 25 mm) and a shorter one (22 mm x 22 mm), separated by two strips of double-sided adhesive tape. Before being assembled into flow cells, the coverslips were washed twice with ethanol, twice with ultrapure water, sonicated for 5 minutes and dried in an oven at ~75 °C.

**Experimental procedure**

A solution of 0.5 mg/mL casein in BRB80 buffer was flowed into a flow cell. After 5 min, the solution was exchanged with the kinesin motor solution (kinesin to coat the surface with 100 ± 20 μm⁻², 500 ± 100 μm⁻² or 1,000 ± 200 μm⁻²; 0.5 mg/mL casein; 0.01 mM or 1 mM ATP),
which in turn was exchanged after 5 minutes with the microtubule solution (16 nM tubulin, 0.5 mg/mL casein, 10 μM paclitaxel; 20 mM D-glucose, 20 μg/mL glucose oxidase, 8 μg/mL catalase, 10 mM dithiothreitol and 0.01 or 1 mM ATP in BRB80). After another 5 min, the microtubule solution was exchanged with an enzymatic antifade solution (0.5 mg/mL casein, 10 μM paclitaxel; 20 mM D-glucose, 20 μg/mL glucose oxidase, 8 μg/mL catalase, 10 mM dithiothreitol and 0.01 or 1 mM ATP in BRB80).44,78 All experiments were performed at 24 ± 1 °C.

Image acquisition and data analysis

The flow cells were imaged using a Nikon TE2000-U epi-fluorescence microscope equipped with an iXON DU897 Ultra electron-multiplying charge-coupled device (EMCCD) camera (Andor) and a 100× oil objective (NA = 1.45). The light source was a Sola light engine (Lumencore). For each flow cell, a field of view was randomly selected and images were taken every 2 or 5 seconds for 30 minutes. The exposure time was 200 ms for all images. Data analysis was conducted using FIESTA filament tracking software79 (available at https://www.bcube-dresden.de/fiesta/wiki/FIESTA) to measure the length and number of microtubules. The average microtubule length was calculated by measuring the length of each microtubule on the first frame it appeared in the field-of-view (in other words, before it starts wearing), and taking the average of that number. The ImageJ imaging software (available at http://rsbweb.nih.gov/ij/) was used to track single detachment events. The number of crossing events was manually counted for one quadrant of the field of view for all images in an experiment. Imaging started less than a minute after adding the antifade solution to the flow cell and all 30 minutes of the video were analyzed.

For the interference reflection microscopy measurements, a 50/50 mirror (Chroma) was used instead of the dichroic mirror as previously described.80 Similar to the fluorescent microscopy...
experiments, the sample was illuminated with a Sola light engine (Lumencore). The gilding assay was performed as described above.

Determining the surface density of kinesin motors

We performed the landing rate experiments\textsuperscript{71,81} to determine the surface density of kinesin. Assuming that the kinesin in solution adsorbs uniformly to the casein-coated glass surface within 5 minutes, we counted the number of microtubules that would absorb on the surface over time at different dilutions of the kinesin stock. We plotted the evolution of this number, and fitted it with the following equation (Figure 8):

\[
N = N_{max} \times (1 - e^{-R*(t-t_{ini})/N_{max}}) \tag{2.5}
\]

Here, \(N\) is the number of microtubules, \(N_{max}\) is the maximum number of microtubules that land for each dilution, \(R\) the landing rate for each dilution, \(t\) the time, and \(t_{ini}\) is the time elapsed before imaging started. The fit parameters were \(N_{max}\), \(R\) and \(t_{ini}\).
Figure 8. Number of microtubule landing events in the experimental field-of-view as a function of time for different dilution factors (ξ) of the stock solution of kinesin.

Then, having the landing rate value for the different dilution, we plotted the landing rate over the dilution factor (Figure 9) and fitted that curve with the following equation:

\[ R = Z \times (1 - e^{(-L^*w^*\rho_0^*\xi)}) \]  \hspace{1cm} (2.6)

Here, Z is a constant, ξ is the dilution factor, ρ₀ represents kinesin surface density obtained when undiluted stock solution of kinesin is used, and L^*w is the interaction area of a microtubule with the surface (with L being the average length of microtubules in each experiment, and w being the width of a microtubule, which was set equal to 20 nm following the findings of Dumont et al.3)
Figure 9. Landing rate of microtubules $R$ as a function of the stock kinesin dilution factor $\xi$.

The fit yields: $\rho_0 = 11,000 \pm 2,000 \mu m^{-2}$

Observation of detaching microtubules

We observed the gliding of fluorescently labeled microtubules on full-length kinesin physisorbed to casein-coated glass surfaces at kinesin densities of 100, 500 and 1,000 $\mu m^{-2}$ and ATP concentrations of 0.01 and 1 mM (resulting in gliding velocities of 70-180 nm/s and 560-700 nm/s). By imaging the microtubules every 5 seconds, detachment events could be directly observed and manually counted.
The microtubules detach after traveling less than a centimeter, rather than maintaining their attachment for the astronomically large distances predicted by the current theories (given in Table 1). The only exception is found at the lowest motor density (100 μm⁻²) and an ATP concentration of 0.01 mM, where the detaching microtubules are also particularly short. In this condition, the theoretically expected gliding distance is actually significantly smaller than the observed gliding distance.

**Types of microtubule detachment**

Two types of detachment events can be observed in all experimental conditions: spontaneous detachment and crossing-induced detachment (Table 5). We define a detachment event as spontaneous when a microtubule leaves the surface without prior deformation due to an inactive kinesin or without another microtubule in its immediate vicinity (Figure 10, parts a to d). Crossing-induced detachment can occur when two microtubules collide and the bottom microtubule serves as a lever inducing the detachment of the second microtubule (Figure 10, part e). Previous studies of the crossing events have shown that 50-100% (depending on motor density) of the crossing microtubules pass over the other microtubule, but then rebind with the kinesin-coated surface on the other side. Here we find that the number of microtubules which fail to reconnect on the other side is small (the percentage of detaching microtubules goes from 0.33% to 10% in different experimental setups) but not zero. We refer to these complete detachments as “crossing-induced detachments”.
Figure 10. Fluorescence microscopy images of microtubule detachment. (a) An entire field of view. (b), (c) and (d):
Spontaneous microtubule detachment – Long (~ 10 µm) (b), medium-length (~ 5 µm) (c), and short (~ 1 µm) (d) microtubules (marked with white arrows) gradually detach and leave the surface. All of the above microtubules are longer than 1 µm. (e): Crossing-induced microtubule detachment – The vertical microtubule gradually detaches as it crosses over another. The kinesin density is 500 µm⁻² and the ATP concentration is 1 mM in all images. (b) is taken from the same frame as (a), but (c), (d) and (e) come from other frames of that experiment. Both scale bars are 10 µm.

Trajectories of detaching microtubules

Detaching microtubules do not have a significantly different trajectory compared to non-detaching microtubules, as shown in Figure 11. More specifically, these trajectories are not particularly curved, and the microtubules do not bend or swivel before detaching from the surface.
Figure 11. Trajectories of detaching microtubules. Each row represents the last 4 frames of the trajectory of a microtubule that spontaneously detached from the surface of the flow cell. The 5th
frame represents the frame on which the microtubule is leaving, or has left the surface. The top 3 microtubules are randomly selected from a high velocity (1 mM ATP) experiment and the bottom 2 microtubules are randomly selected from a low velocity (0.01 mM ATP) experiment. The kinesin density is of 100 µm⁻¹ in all cases. The white arrows point at the leaving microtubule.

**Calculating the average gliding distance before detachment**

To calculate the average gliding distance before detachment \( \langle d_O \rangle \) (given in the last column of Table 5), for each experiment, we selected 10 microtubules at random in the first frame, tracked their movement for the next 10 frames, and determined the average gliding velocity in this experiment \( v_{Avg} \). We checked that this velocity remained constant in all experiments.

The software FIESTA provides the number of microtubules that are detected in each field of view, as well as the number of frames each microtubule stays in the field of view. Knowing this, we deduced the total gliding distance by all the microtubules \( L_{Total} \) during each experiment (see Table 2) using the equation:

\[
L_{Total} = v_{Avg} \times t \times \sum n_i
\]  \hspace{1cm} (2.7)

where \( t \) is the time elapsed between the images (2 s or 5 s), and \( n_i \) is the number of frames the microtubule \( i \) was present in the field of view.

Lastly, we calculated the average gliding distance before detachment \( \langle d_O \rangle \) using the equation:
\[ \langle d_O \rangle = \frac{L_{Total}}{N_l} \]  \hspace{1cm} (2.8)

Where \( N_l \) is the number of leaving events per experiment (counted manually). The results are reported in Table 5.

**Table 2. Total glided distance of all microtubules in each experimental setup.**

<table>
<thead>
<tr>
<th>Kinesin Density</th>
<th>[ATP]</th>
<th>( L_{Total} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 ( \mu \text{m}^2 )</td>
<td>1 mM</td>
<td>80 ± 10 mm</td>
</tr>
<tr>
<td></td>
<td>10 ( \mu \text{M} )</td>
<td>30 ± 6 mm</td>
</tr>
<tr>
<td>500 ( \mu \text{m}^2 )</td>
<td>1 mM</td>
<td>120 ± 20 mm</td>
</tr>
<tr>
<td></td>
<td>10 ( \mu \text{M} )</td>
<td>30 ± 5 mm</td>
</tr>
<tr>
<td>100 ( \mu \text{m}^2 )</td>
<td>1 mM</td>
<td>270 ± 20 mm</td>
</tr>
<tr>
<td></td>
<td>10 ( \mu \text{M} )</td>
<td>20 ± 4 mm</td>
</tr>
</tbody>
</table>

**Kinesin density contributes to the frequency of spontaneous detachment events**

Our experiments show that the kinesin density significantly contributes to the frequency of spontaneous detachment events (Table 5): student’s t-tests show that differences in kinesin density induce statistically significant different gliding distances before detachment at the 5% level (Figure 12), whereas differences in gliding velocity only affect the detaching rates of lower kinesin density experiments \(^{64} \) (100 \( \mu \text{m}^2 \)). Even though we prove that average glided distance before desorption does depend on the kinesin density, knowing that the desorption rates of microtubules are exponentially distributed (as described later in this Chapter), this distance would not be the best metric to study microtubule detachment.
No obstacle is causing the detachment of microtubules

To understand what causes spontaneous detachment events, we first hypothesized that an obstacle on the surface induces the microtubules to leave the surface, replicating the mechanism for crossing-induced detachment (Figure 10, part e). We had to reject this hypothesis for four reasons:
(1) The surface of the cleaned coverslips is flat

A plausible cause for the spontaneous detachment of microtubules is that an elevated region is present on the surface the flow cell, serving as a lever for the microtubules to take-off and return in solution. To rule out this hypothesis, we first cleaned the surface of our coverslips following the protocol described in the Methods section. Then we characterized 10 randomly chosen 1 mm lines of the surface of several clean coverslips using a profilometer (Figure 13). We analyzed ten 1 mm lines because their sum corresponds to 10 mm, which is larger than the observed average gliding distance before detachment of a microtubule (0.3 – 8 mm) for all conditions tested. We plotted the difference in height along the randomly selected lines on the coverslip, which never exceeded an absolute value of 15 nm. We also determined an RMS value of 4 nm. Since a microtubule glides 17 ± 2 nm above the surface for this type of kinesin according to Kerssemakers et al., it is highly unlikely that the microtubule will encounter a large enough obstacle to cause detachment.

![Figure 13. Height profile of a 1 mm segment of a cleaned coverslip. Left – Height profile (in µm) over a distance of 1 mm on](image-url)
a clean coverslip. *Right – Zoomed-in view of the difference in height (in nm), for the portion of the left curve that is located inside the dotted rectangle.*

(2) The detachment sites are non-deterministic

A key characteristic of the observed detachment events was the non-deterministic nature of the detachment sites: for each detachment site, only one microtubule which glided over that specific location would detach from the surface. In particular, this contrasts with some breaking sites being deterministic and causing more than one microtubule to break (as explained in Chapter 4). Thus, it appears that detachment is not triggered by crossing a specific site on the surface.

(3) Detachment events are randomly distributed on the surface of the flow cell

We studied the spatial distribution of the detachment events to determine whether the detachment sites were randomly distributed across the surface of the flow cell. If this distribution is not random, that would prove that there are sites that are more favorable for microtubule detachment.

For this analysis, we successively divided our 80 µm x 80 µm field of view into 1, 4, 16, 64 and 256 square bins of equal size. After defining the position of each detachment event, the number of events occurring in each bin of each size was computed. Using this data, we plotted, using a logarithmic scale, the magnitude of the standard deviation ($\sigma$) against the mean number of events per bin ($\mu$) for each subsystem size (Table 3 and Appendix, part 1). As described by Narayan et al., a slope of 1 for these logarithmic regressions would prove that there is a dependency
between the regions of the coverslip and the detachment events. In other words, this would mean that there are areas that are more favorable for microtubule detachment along the surface of the coverslip. Thus, all of the computed slopes being statistically significantly different than 1, we can deduce that the locations of leaving events are uniformly distributed on the surface. In other words, detachment events occur at random locations in the field of view.

**Table 3. Statistical analysis of the spatial distribution of detachment events.** This table provides, for all experimental setups, the slope of logarithm of the standard deviation (σ) of detachment events plotted against the logarithm of the mean number of events per bin (µ), as the size of the bins increase. In the fourth column, R² is the coefficient of determination associated with each linear regression.

<table>
<thead>
<tr>
<th>Kinesin density</th>
<th>[ATP]</th>
<th>Log(σ/µ)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 μm²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM</td>
<td>0.57 ± 0.05</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>10 μM</td>
<td>0.52 ± 0.05</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>500 μm²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM</td>
<td>0.48 ± 0.1</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>10 μM</td>
<td>0.53 ± 0.03</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>100 μm²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM</td>
<td>0.57 ± 0.1</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>10 μM</td>
<td>0.57 ± 0.2</td>
<td>0.90</td>
<td></td>
</tr>
</tbody>
</table>
(4) There are no obstacles on the surface during the assay

Using the method described by Mahamdeh et al., Gadiel Saper used interference reflection microscopy (IRM) to image kinesin-propelled microtubules in conditions similar to those of the experiments described above (kinesin density: 500 µm\(^{-2}\) and ATP concentration: 1 mM). This experiment proved that:

- Our cleaning method is effective in removing dust particles from the surface of the coverslip, and no additional particle of the same size as the microtubules is introduced during the flowing-in process (Figure 14, top).

- Obstacles are not at the origin of detachment events, since we can observe detachment events in clean surfaces and in absence of any visible obstacle (Figure 14, bottom).
Figure 14. Gliding microtubules imaged using IRM. *Top* – Microtubules gliding on an uncleaned and a cleaned coverslip. In the uncleaned coverslip, the dark spots represent dust particles that are present on the surface. On the cleaned coverslip, no spot is visible, showing that no particle of that size is present on the surface. The only dark spot on that image corresponds to a dead pixel on the camera. *Bottom* – Last 3 frames before detachment and 1st frame after detachment from the clean glass surface of a ~ 10 µm microtubule (pointed at by the white arrows). Scale bars are 10 µm.
Defective kinesin-bound microtubules do not cause the detachment events

While we did observe interruptions in the smooth gliding of the microtubules, presumably due to the attachment to defective kinesins, these events were not correlated with the observed detachment events. However, these events are correlated to the breaking rates of microtubules (see next chapter). The breaking and wearing rates of microtubules explain the observed differences in the average length of microtubules in our experiments even though all experiments were performed using the same batch of polymerized microtubules.

The detachment rate increases as time passes

Similarly, we studied the temporal distribution of the detachment events to determine whether the detachment events were randomly distributed in time. If not, that would prove that there are moments in the experiment that are more favorable for microtubule detachment.

For this analysis, the total time elapsed in each recording was split into 2, 4 and 8 bins of equal size. Each bin represents a period of time in the recording, in which the total number of leaving events were recorded along with the average number of microtubules present during the time period. A chi-squared test was performed to compare the expected number of leaving events for each time period given a certain number of microtubules and the observed number of leaving events (See Appendix, part 2). For all experiments with enough detachment events to conduct statistical analyses, we found a statistically significant difference between the expected and the observed detachment patterns, proving that detachment events are not randomly distributed in time (Figure 15). More specifically, the frequency of detachment events increases exponentially with
time for high velocity experiment. Details regarding the statistical testing are given in Table 4 as well as at the end of this chapter.

Figure 15. Evolution of the leaving rate per microtubule over time. The leaving rate follows an exponential increase for experiments with 1mM ATP concentration. The crosses represent the experimental data and the lines represents their fits. The parameters of the fitted lines are given in Table 4. Experiments with ATP concentrations of 10 µM have not been included because of the limited number of detachment events for the 2 conditions with higher kinesin densities. In order to give equal weight to all
experimental data points, the logarithm of the leaving rate was fit using a linear regression.

**Table 4. Statistical analysis of the temporal distribution of detachment events and fitted detachment rates.** This table provides, for all experimental setups, the results of the chi-squared test on the time distribution of the detachment events. N/A indicates insufficient data points for conducting the statistical analysis. The large standard deviations of the fit parameters for the 1 mM ATP concentration and 100 μm² kinesin density experiment are due to the limited number of data points available to do the regression.

<table>
<thead>
<tr>
<th>Kinesin density (μm²)</th>
<th>[ATP]</th>
<th>χ² test</th>
<th>Fitted values to the equation: ( \log_{10}(\lambda_s) = at + b )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>where ( \lambda_s ) is the detachment rate, ( t ) is the time, and ( a ) and ( b ) are the fit parameters.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.05?</td>
<td>a ± S.D. (s⁻¹)</td>
</tr>
<tr>
<td>1000</td>
<td>1 mM</td>
<td>Yes</td>
<td>8.0<em>10⁻⁴ ± 1.5</em>10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>10 μM</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>500</td>
<td>1 mM</td>
<td>Yes</td>
<td>9.7<em>10⁻⁴ ± 4.5</em>10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>10 μM</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>100</td>
<td>1 mM</td>
<td>Yes</td>
<td>10.1<em>10⁻⁴ ± 7.5</em>10⁻⁵</td>
</tr>
</tbody>
</table>
Rewriting the equation that was fit with an exponential function, we get:

\[ \lambda_3(t) = 10^{at} \times 10^b \quad (2.9) \]

The values that we get for \(10^b\) in the 3 experimental setups scale with \(\frac{1}{\sigma^2}\), \(\sigma\) being the kinesin density of the experiment. The density \(\sigma\) is itself proportional to the linear density \(q\) of kinesin, and we have, for a microtubule of length \(L_{MT}\):

\[ N = L_{MT} \times q \quad (2.10) \]

where \(N\) is the number of kinesin motors that are attached to a microtubule. As a result, according to the results presented in Table 4, the detachment rate of microtubules is proportional to \(\frac{1}{N^2}\), which is unexpected since the theoretically estimated \(64\) average travel distances of microtubules scale with \(k^N\) where \(k\) is a constant.

Photodamage could be the cause of the observed phenomenon: as the microtubules that are in the field-of-view suffer from photodamage, free radicals would be generated in the vicinity of the fluorescently-labeled microtubules, damaging some of the motors. As a result, detachments become more likely to occur as the experiment goes on, since more motors get damaged with time.
Table 5. Observed detachment events, crossing events, gliding velocity, and calculated average gliding distance before detachment for the different experimental conditions.

<table>
<thead>
<tr>
<th>Kinesin density (μm⁻²)</th>
<th>ATP concentration (mM)</th>
<th>Average length of microtubules (μm) ± S.D.</th>
<th>Average gliding velocity (nm/s) ± S.D.</th>
<th>Number of observed microtubules</th>
<th>Number of observed detachment events in 1800 s of imaging</th>
<th>Average length of detaching microtubules (μm) ± S.D.</th>
<th>Average gliding distance before spontaneous detachment 〈d₀〉 (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>1</td>
<td>1.7 ± 1.7</td>
<td>560 ± 20</td>
<td>1506</td>
<td>116</td>
<td>1.0 ± 0.7</td>
<td>7 ± 2</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>5.3 ± 5.4</td>
<td>180 ± 20</td>
<td>1095</td>
<td>76</td>
<td>1.5 ± 1.2</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>500</td>
<td>1</td>
<td>3.9 ± 3.3</td>
<td>670 ± 20</td>
<td>1178</td>
<td>125</td>
<td>1.2 ± 0.4</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>5.9 ± 6.0</td>
<td>70 ± 6</td>
<td>940</td>
<td>77</td>
<td>2.8 ± 1.6</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td>2.6 ± 2.4</td>
<td>700 ± 60</td>
<td>741</td>
<td>38</td>
<td>2.4 ± 1.0</td>
<td>0.3 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>4.7 ± 4.9</td>
<td>110 ± 6</td>
<td>1119</td>
<td>65</td>
<td>7.7 ± 1.4</td>
<td>1 ± 0.2</td>
</tr>
</tbody>
</table>
Microtubules could be “curling” away” from the surface

The theoretically considered effects of a random distribution of defective kinesins and a limited run length of the kinesins do not fully explain the observed average gliding distances, especially since we observed the detachment of microtubules at the beginning of all experiments.

A sensible hypothesis would be that the spontaneous detachment of microtubules originates from a “curling away” from the surface of the flow cell as they move. Gosselin et al. suggested that microtubules have a preferred curvature, being either in a straight state; in a weakly curved state with radius of curvature of 10 µm; or a highly curved state with radius of curvature of 1 µm. As a result of these states, microtubules move on the surface in circular patterns rather than in a persistent random walk. However, if a microtubule assumes one of its curved states, it will move away from the surface of the flow cell. It would be gradually held by fewer and fewer kinesins, and it would eventually fully detach from the surface. The same mechanism may be responsible for crossing-induced detachment events, only instead of the initial detachment of the tip of the microtubule occurring randomly, it is caused by the “bottom” microtubule forcing the other one’s tip to detach in order to be able to glide across it. Figure 16 summarizes the different hypotheses considered in our study of microtubule detachment.

One should also keep in mind that the theories exposed in this Chapter are also proven for very low densities of kinesins, with an average of 2 to 4 kinesins being bound to a microtubule. These theories, although valid at those densities, might also need to be adapted to high kinesin densities.
Figure 16. Potential causes of spontaneous microtubule detachment. (a): An obstacle on the surface leads to microtubule detachment, which we disproved. (b): A local depletion of kinesin motors provides microtubules with the opportunity to diffuse away from the surface and detach, which is disproved by the theories exposed in this Chapter. (c): Kinesin motors get damaged over time
and fail to attach the microtubules to the surface. (d): The microtubule spontaneously detaches from the surface of the flow cell by “curling away” from it.

**Conclusion and future directions**

Microtubule detachment is a non-negligible phenomenon in inverted motility assays, limiting average gliding distances before detachment to a few millimeters. The frequency of detachment events depends on the kinesin density, and increases exponentially with time. Microtubule detachments are many orders of magnitude more frequent than expected in assays with a high kinesin density based on prior theoretical work. These observations must be taken into account for the design of nanodevices incorporating cytoskeletal filaments propelled by surface-adhered biomolecular motors.

The next research direction to take as follow-up this project relies on taking advantage of the IRM setup. For instance, the effect of photobleaching could be studied more in-depth: there is no need to use fluorescence microscopy with the IRM method, so we can now fully remove photobleaching as a source of degradation. By analyzing videos in similar experimental conditions as those described in this Chapter, one could determine the detachment rate of microtubule in the absence of fluorescence, and the evolution of this rate through time. If the detachment rate remains constant, it would prove that the fluorescent is at the origin of the observed exponential increase in the detachment rate. This could be caused either by the degradation or the shrinking of the microtubules, or by the fluorescent light damaging the kinesin motor proteins.
1. Statistical analysis – Detachment events are uniformly distributed in space

For this analysis, we divided our field of view into 1, 4, 16, 64 and 256 square bins of equal size. After defining the position of each detachment event, the number of events occurring in each bin of each size was computed. The table below gives the mean number of events per bin ($\mu$) as well as standard deviation ($\sigma$) for each subsystem size. Only spontaneous detachment events have been taken into account for this analysis.

**Appendix Table 1. Mean and standard deviation of the number of microtubules per bin for different bin sizes.**

<table>
<thead>
<tr>
<th>Kinesin density ($\mu$m$^2$)</th>
<th>ATP concentration (mM)</th>
<th>$\mu \pm$ S.D. of the number microtubule per bin when the field-of-view is divided between the given number of bins</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>1</td>
<td>$11 \pm 0$  $2.8 \pm 2.4$  $0.7 \pm 1.0$  $0.2 \pm 0.5$  $0.04 \pm 0.2$</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>$4 \pm 0$  $1.0 \pm 1.9$  $0.25 \pm 1.1$  $0.06 \pm 0.5$  $0.02 \pm 0.2$</td>
</tr>
<tr>
<td>500</td>
<td>1</td>
<td>$39 \pm 0$  $9.8 \pm 4.4$  $2.4 \pm 1.9$  $0.6 \pm 1.1$  $0.2 \pm 0.5$</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>$8 \pm 0$  $2 \pm 1.5$  $0.5 \pm 1.2$  $0.13 \pm 0.7$  $0.03 \pm 0.3$</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td>$70 \pm 0$  $17.5 \pm 4.3$  $4.4 \pm 1.3$  $1.1 \pm 3.0$  $0.27 \pm 1.2$</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>$19 \pm 0$  $4.8 \pm 1.3$  $1.2 \pm 1.8$  $0.3 \pm 0.9$  $0.07 \pm 0.4$</td>
</tr>
</tbody>
</table>
2. Statistical analysis – Detachment events are not uniformly distributed over time

Below are a series of tables that contain all the statistical tests used. For each combination of kinesin density and ATP concentration tested, we performed several chi-squared tests. To do so, we determined the number of leaving events, as well as the total number of unique microtubules that appear in frame during certain time interval. This information is found in the tables labeled “Observed”. We then calculated the expected frequency of leaving events in each time frame, which is found in the tables labeled “Expected”. For many of our experimental setups, we performed multiple chi-squared tests using differing sizes of time frames, from 2 to 8 equally sized bins. This was done to confirm that the selected bins would not influence the results of the chi-squared test. The results of each chi-squared test performed is listed in tables labeled results under their respective experimental conditions.

In the tables, the “Expected” columns refer to the expected number of detaching and non-detaching microtubules based on the number of experimentally observed detachments per experiment. The overall total number of microtubules is higher than the number reported in Table 5 because some microtubules “overlap” time bins and are present in 2 consecutive bins. Thus, those microtubules are counted twice.

The experiments with an ATP concentration of 10 µM and a kinesin density of 1000 μm⁻² and 500 μm⁻² respectively have 4 and 8 detachment events. This number of detachment events is not high enough to conduct conclusive statistical tests. Hence, these experiments are not represented in the tables below.
Appendix Table 2. Data used to perform the chi-square test.

Experiment with a kinesin density of 1000 μm\(^2\) and an ATP concentration of 1 mM.

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Observed</th>
<th>Expected</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Detaching microtubules</td>
<td>Non-detaching microtubules</td>
<td>Detaching microtubules</td>
</tr>
<tr>
<td>For 2 groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-905</td>
<td>7</td>
<td>1258</td>
<td>13</td>
</tr>
<tr>
<td>906-1811</td>
<td>9</td>
<td>249</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
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<td>1507</td>
<td>16</td>
</tr>
<tr>
<td>For 4 groups</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1-453</td>
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<td>10</td>
</tr>
<tr>
<td>454-905</td>
<td>3</td>
<td>311</td>
<td>3</td>
</tr>
<tr>
<td>906-1359</td>
<td>6</td>
<td>147</td>
<td>2</td>
</tr>
<tr>
<td>1360-1811</td>
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<td>113</td>
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</tr>
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<td>16</td>
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Results

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<th>$\chi^2_{\text{crit}}$</th>
<th>P &lt; 0.05?</th>
</tr>
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<td>2</td>
<td>19.18</td>
<td>3.84</td>
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</tr>
<tr>
<td>4</td>
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<td>7.82</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Appendix Table 3. Data used to perform the chi-square test.

Experiment with a kinesin density of 500 μm$^{-2}$ and an ATP concentration of 1 mM.

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<th></th>
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<tr>
<td></td>
<td>Observed</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Non-detaching microtubules</td>
<td>Detaching microtubules</td>
<td>Non-detaching microtubules</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>For 2 groups</td>
<td>1-905</td>
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<td>60</td>
<td>974</td>
<td>1034</td>
</tr>
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<td>122</td>
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<td>144</td>
<td>153</td>
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<td>69</td>
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<table>
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<th></th>
<th></th>
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</tr>
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<td>Detaching microtubules</td>
<td>Non-detaching microtubules</td>
<td>Detaching microtubules</td>
<td>Non-detaching microtubules</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>For 4 groups</td>
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<tr>
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<td>Detaching microtubules</td>
<td>Non-detaching microtubules</td>
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<td>37</td>
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</tr>
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<td>1</td>
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<td>1</td>
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**Results**

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<th>$\chi^2_{crit}$</th>
<th>P &lt; 0.05?</th>
</tr>
</thead>
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<tr>
<td>8</td>
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Appendix Table 4. Data used to perform the chi-square test.

Experiment with a kinesin density of 100 μm\(^2\) and an ATP concentration of 1 mM.

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<td>Detaching microtubules</td>
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</tr>
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</table>

For 2 groups

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<td>39</td>
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</tr>
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## For 8 groups

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## Results

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<th>$\chi^2_{\text{crit}}$</th>
<th>$P &lt; 0.05?$</th>
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Appendix Table 5. Data used to perform the chi-square test.

Experiment with a kinesin density of 100 μm⁻² and an ATP concentration of 10 µM.

<table>
<thead>
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<th>Observed</th>
<th>Expected</th>
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<td>Non-detaching microtubules</td>
<td>Detaching microtubules</td>
</tr>
<tr>
<td>For 2 groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-905</td>
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<td>906-1811</td>
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<td>18</td>
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<td>Total</td>
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</tr>
<tr>
<td>For 4 groups</td>
<td></td>
<td></td>
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</tr>
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</tr>
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<td>Total</td>
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<td>Number of Groups</td>
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<td>$\chi^2_{crit}$</td>
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<td>5.73</td>
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</tr>
<tr>
<td>4</td>
<td>17.58</td>
<td>7.82</td>
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</table>
Chapter 3.

Microtubule wear, detachment and breaking combine to mechanically degrade *in vitro* kinesin-powered molecular shuttles

Introduction

Microtubule breakage is not a newly-discovered mechanism. In 1997, Waterman-Storer and E.D. Salmon observed the breakage of 7 microtubules *in vivo*. The breakages were caused by the bending of the microtubules, with an average radius of curvature at the breaking point of $0.6 \pm 0.15 \, \mu\text{m}^{-1}$. Two years later, Odde et al. studied the bending and breaking of microtubules in living fibroblast cells. They proved that breaking is likely a mechanism causing the creation of noncentrosomal microtubules, and they suggested that microtubules played an important role in the mechanochemical information processing of cells.

Rather, recently, the study of microtubule mechanics both *in vitro* and *in vivo* has emphasized two main areas. First, the self-repairing properties of microtubules have been investigated following the demonstration by Schaedel et al. that microtubule dynamics do not exclusively occur at their ends since microtubules can “self-heal” and adapt to mechanical stresses. Secondly, the mechanical properties have been determined with a higher degree of
precision: Kabir et al. have determined the Young’s modulus of immobilized microtubules by breaking them through the application of controlled tensile stresses and they revealed that the interactions between microtubules and kinesin can modulate the rigidity of the microtubules.93

However, in vitro, even though microtubule breakages have been observed,44,51 no study has looked into the different causes and consequences of microtubule breakage in activated molecular shuttles yet. The breakages shorten the molecular shuttles’ lifetime, thus limiting the applications they could be used for.

Another motivation for our study is that microtubule breakage is one of the sources of molecular shuttle degradation: wear and detachment fail to fully describe the experimentally observed in vitro degradation of kinesin-powered molecular shuttles. Indeed, in their study of the lifetime of molecular shuttles, Brunner et al.44 observed an initial increase in microtubule number followed by a decay of that number, and Yoli Jeune-Smith made the same observation a few years later.51 If shrinking and detachment were the only degradation mechanisms in molecular shuttles, the number of microtubules would not increase initially: it would either remain constant or slightly decrease due to the detachment events. Thus, they initial increase in the number of microtubules indicates that breaking is another one of the degradation mechanisms acting on molecular shuttles.

Lastly, a study similar to ours has been conducted on actin filaments: Arai et al.94 continuously controlled the radius of curvature of an actin filament by tying a knot in it, using optical tweezers to manipulate the filament. Through this experiment, they found that actin filaments break at the knot when the radius falls below 0.2 µm, which corresponds to a pulling force at breakage of around 1 pN. As a result, our study on microtubule breakage will give us the opportunity to gain an additional knowledge regarding the mechanisms regulating the mechanics of cytoskeletal filaments as an ensemble.
Here, we provide detailed measurements of an active nanosystem that displays degradation as a result of its mechanical activity. Our goal is to design a model that takes into account all the mechanisms leading to the degradation of molecular shuttles \textit{in vitro}, which we believe are: microtubule shrinking, detachment and breakage. By understanding how this system works, future investigations can work on increasing its lifetime, increasing its range of applications.

\textbf{Materials and Methods}

The methods and materials used for the microtubule polymerization, the preparation of the kinesin solution, the flow cells, as well as the experimental procedure, the image acquisition and the data analysis are described in Chapter 2.

\textbf{Types of microtubule breaking}

Using the same experimental data as for Chapter 2, we directly observed and manually counted more than 150 breaking events (Table 6). The microtubule breaking rate is correlated with both the kinesin density and the velocity of the microtubules.

We observed two types of breaking events: background breaking and high-curvature-induced breaking. Background breaking occurs while a microtubule sustains its smooth gliding and breaks without encountering any obstacle on its path (Figure 17, Top). This contrasts with high-curvature-induced breaking, during which a microtubule gets stuck on the surface due to the presence of an obstacle (most probably a defective kinesin\textsuperscript{76} motor or a dust particle) on the surface, bends, and breaks due to the strain the tubulin dimers get subjected to (Figure 17, Bottom). More examples of breaking events are provided in the Appendix (Part 1).
Table 6. Observed breaking events, crossing events, gliding velocity, and mean curvature at the breaking sites for the different experimental conditions.

<table>
<thead>
<tr>
<th>Kinesin density (μm⁻²)</th>
<th>ATP concentration (mM)</th>
<th>Average gliding velocity (nm/s) ± S.D.</th>
<th>Number of observed microtubules</th>
<th>Number of observed breaking events</th>
<th>Mean curvature (μm⁻¹) ± S.D. at breaking sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Background</td>
<td>High-curvature-induced</td>
</tr>
<tr>
<td>1000</td>
<td>1</td>
<td>560 ± 20</td>
<td>1506</td>
<td>7</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>180 ± 20</td>
<td>1095</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>500</td>
<td>1</td>
<td>670 ± 20</td>
<td>1178</td>
<td>3</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>70 ± 6</td>
<td>940</td>
<td>1</td>
<td>31</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td>700 ± 60</td>
<td>741</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>110 ± 60</td>
<td>1119</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
</tr>
</tbody>
</table>
Figure 17. The 2 types of breaking events. Top – Background breaking: the microtubule breaks without any out-of-the-ordinary strain being applied to its lattice. Bottom – High-curvature induced breaking: the microtubule encounters an obstacle on the surface and strongly bends (4th image), exerting a high strain on its lattice, which leads to the microtubule breaking at the site of its highest curvature. In the 4 first images of both rows, the white arrows point at the “mother” microtubules that breaks into 2 daughter microtubules in the 5th image (pointed at by the 2 white arrows on that image). Both scale bars are 10 µm.
Causes of microtubule ruptures

Background breaking

As formulated by Guo et al., a sequential breaking of the protofilaments of a microtubule could be at the origin of microtubule rupture. Hence, background breaking could result from the gradual weakening of a microtubule due to the repeated forces exerted by kinesin motors on the microtubule, added to the photodamage experienced while the microtubule is in the field-of-view. The breakage of the microtubule shown in Figure 18 corroborates this hypothesis: the pictured microtubule has defects on its lattice that have led to the existence of a visibly weakened site (Figure 18, Top). The microtubule bends with high curvature and eventually breaks at that site (Figure 18, Bottom right) without encountering any obstacle on its path.

High-curvature-induced breaking

More than 90% of the observed breakages were caused by strong bending of microtubules after they encountered a defective kinesin on their path. To show how high curvatures affect the rupture of microtubules, we calculated the curvatures along the path of 20 randomly chosen microtubules in each experimental condition (defined as a combination of ATP concentrations of 1 mM and 10 µM, and kinesin densities of 1000 µm⁻², 500 µm⁻² and 100 µm⁻² – see Appendix, part 2 for more information). Since we did not observe any significant difference in the distribution of breaking curvatures in the different experimental conditions, the rest of the study will include all the collected experimental data.
Figure 18. **Microtubules with defects in their lattice will break at their weakened site.** *Top* – Microtubule with a defect in its lattice. The defective site is pointed at by the white arrow, and is recognizable by the combination of: (1) bending with unexpected curvatures, and (2) a dimmer intensity, hinting at the fact that (fluorescent) tubulin dimers are missing from the microtubule lattice. *Bottom* – Last 3 frames before breakage and 1st frame after breakage of that microtubule. The white arrows point at the defective site of the microtubule and the orange arrows point at the 2 “daughter” microtubules. The images are drawn from the
experiment using 1 mM ATP and a kinesin density of 500 µm⁻². The scale bar is 10 µm.

Using a logistic regression to find the critical breaking curvature \( \kappa_c \) of microtubules

According to Zhurkov,⁹⁶ the relation between lifetime, stress and temperature can be written under the form:

\[
\tau = \tau_0 e^{\frac{(U_0 - \gamma \sigma)}{kT}}
\]  
(3.1)

where \( \tau_0 \) is the reciprocal of the natural oscillation frequency of atoms in the solid, \( U_0 \) is the binding energy on the atomic scale, \( \gamma \) is a constant coefficient for each solid, depending on its molecular structure, \( \sigma \) is the stress, \( k \) is Boltzmann’s constant and \( T \) is the temperature. Since the temperature is constant in our experiment, equation (3.1) can be rewritten as:

\[
\tau = \tau_0' e^{\frac{U_0}{kT} - \frac{\gamma \sigma}{kT}} = \tau_0'e^{-\frac{\sigma}{\sigma^*}}
\]  
(3.2)

with \( \tau_0' = \tau_0 e^{\frac{U_0}{kT}} \) and \( \sigma^* = \frac{kT}{\gamma} \).

As a result, the breaking rate is:

\[
\lambda = \lambda_0 e^{\frac{\sigma}{\sigma^*}}
\]  
(3.3)

with \( \lambda = \frac{1}{\tau} \) and \( \lambda_0 = \frac{1}{\tau_0'} \).

Considering that the breaking rate does not depend on time, that is to say that the history of the system does not affect the breaking rate, the probability \( P_b(\Delta t) \) of breaking in a time \( \Delta t \) is of (see proof in the 4th part of the Appendix):

\[
P_b(\Delta t) = 1 - e^{-\lambda t}
\]  
(3.4)
which can be rewritten as

$$P_b(\Delta t) = \left(1 - e^{-\lambda \Delta t}\right) * e^{\lambda \Delta t} / e^{\lambda \Delta t} = \frac{e^{\lambda \Delta t} - 1}{e^{\lambda \Delta t}}$$ (3.5)

For small values of $\lambda \Delta t$, the Taylor expansion of equation (3.5) gives:

$$P_b(\Delta t) = \left(\frac{1 + \lambda \Delta t - 1}{1 + \lambda \Delta t}\right) = \frac{\lambda \Delta t}{1 + \lambda \Delta t}$$ (3.6)

which becomes, using equation (3.3):

$$P_b(\Delta t) = \frac{\lambda_0 e^\theta / \sigma^*/\Delta t}{1 + \lambda_0 e^\theta / \sigma^*/\Delta t} = \frac{e^{a+\sigma+b}}{1 + e^{a+\sigma+b}}$$ (3.7)

with $a = 1 / \sigma$ and $b = \ln(\lambda \Delta t)$.

Equation (3.7) is the typical form of a logistic regression. Furthermore, for small forces, the beam equation can be simplified into:

$$M = EI \kappa$$ (3.8)

where $M$ is the bending moment, $E$ is Young’s Modulus, $I$ is the second moment of inertia and $\kappa$ is the curvature. In addition to that, the formula for the bending stress states that:

$$|\sigma| = \frac{My}{I}$$ (3.9)

where $\sigma$ is the bending stress and $y$ is the distance from the middle of the section. By combining equations (3.8) and (3.9), we get:

$$|\sigma| = Ek \kappa y$$ (3.10)

In other words, the stress and the curvature are proportional, so we can do our logistic regression on the curvatures of the microtubules. Based on this reasoning, we obtained the distribution of curvatures along 120 microtubule paths that were randomly selected amongst all experimental conditions, as well as the curvature distribution at the breaking sites (Figure 19, Bottom) of the microtubules. Then, using a logistic regression, we were able to find the critical
The curvature $\kappa_c$ defined as the curvature for which the breaking probability of the microtubule is of 0.5 (Figure 19, Top).

The logistic regression fit gives: $\kappa_c = 0.16 \pm 0.01 \mu m^{-1}$

From the critical curvature $\kappa_c$ to the critical force $F_c$

Knowing that stress is obtained by dividing a force by an area, we can rewrite equation (3.10) as:

$$|F| = E\kappa yA$$  \hspace{1cm} (3.11)

where $F$ is the force exerted on the microtubule, and $A$ is the area on which the force is exerted. So, the critical force exerted on a microtubule subject $\kappa_c$, with a Young’s modulus of 1.9 GPa, at a distance from the middle section of 10 nm (chosen as the average of the inner and outer radii of a microtubule), and $A$ being the cross-sectional area of a tubulin dimer, is of:

$$|F_c| = 60 \pm 3 \mu N$$
**Figure 19. Logistic regression and distribution of curvatures.**

*Top* – The curvatures of the gliding microtubules have been assigned a y-value of 0, and the curvatures of the microtubules at their breaking sites have been assigned a y-value of 1. The orange curve represents the fitted curve to the logistic equation. $\kappa_c$ is the point where the y-value of the fit is equal to 0.5. *Middle* – Curvatures along the paths of 120 randomly chosen microtubules. *Bottom* – Curvatures at the observed breaking points. The middle and bottom graph show that there is a significant shift in the distribution of curvatures between the gliding microtubules and their breaking sites, the curvatures of the breaking sites being on average an order of magnitude higher than the curvatures of the gliding microtubules. The red line represents the critical curvature $\kappa_c$ at which the probability of microtubules breaking is of 0.5.

**Implications of the critical curvature $\kappa_c$ and of the critical force $F_c$**

The force exerted by a kinesin motor on a microtubule at each step it takes is of 5 pN. The critical force is an order of magnitude higher, showing that it would take the combined action of at least 10 kinesin motors to cause the breakage of a microtubule.

Memet et al. demonstrated that microtubules soften when they experience high strains due to their cross-sectional ovalization and eventual buckling, an effect first described for macroscopic hollow cylinders by Brazier in 1927. Memet et al. calculated a critical curvature at the onset of microtubule buckling of between 0.1 and 0.2 $\mu$m$^{-2}$. In their experiment, the microtubule was
held in place and continuously bent using optical trapping. Our results fall into the boundaries they set, proving that (1) the mechanical behavior of gliding and immobilized microtubules is the same, and (2) the mechanical principles that are valid at the macroscale are transposable at the micro- and nanoscale.

When looking at the mechanics of cytoskeletal filaments more in general, Arai et al. found that actin filaments break when the pulling force at breakage is of 1 pN, whereas the critical force to break a microtubule is of 60 pN. The difference between these forces is explained by the difference in the bending moment of the two filaments. Indeed, an actin filament modeled as a homogenous isotropic elliptical cylinder with a major radius of 4.5 nm and mean radius of 3.5 nm has a second moment of inertia \( I_{\text{actin}} \) that can be written according to equation (3.12).

\[
I_{\text{elliptical cylinder}} = \frac{\pi r_a^2 r_b^2}{4}
\]  

(3.12)

So: \( I_{\text{actin}} = 120 \text{ nm}^4 \)

where \( r_a \) is the major radius and \( r_a \) is the minor radius of the elliptical cylinder.

On the other hand, microtubules have a flexural rigidity of 26*10^{-24} N.m², and a Young’s modulus of 1.9 GPa. So their second moment of inertia \( I_{\text{microtubule}} \) is equal to:

\[
I_{\text{microtubules}} = \frac{EI}{E} = 1.4 \times 10^{-33} \text{ m}^4
\]

\( I_{\text{microtubules}} = 1400 \text{ nm}^4 \)

In other words, microtubules are 12 times harder to bend than actin filaments, which explains why they need a force more than an order of magnitude higher than actin filaments to be broken.
Monte-Carlo simulations show that shrinking, detachments and breakage cause the *in vitro* degradation of molecular shuttles

We designed a Monte-Carlo simulation to model the degradation of the original distribution of microtubules used for the experiments, taking into account both the decline in the number and the decline in the length of microtubules. Based on each experimental condition, constant shrinking and breaking rates, and an exponentially increasing detachment rate were set. If as a result of breaking or shrinking, a microtubule got shorter than a set cut-off length, it was not considered in the simulation anymore. The cut-off length that was used in the FIESTA software (from which we obtained both the experimental lengths and the experimental number of microtubules per frame) was of 0.5 µm, the cut-off length was set to 0.5 µm for the simulations as well. The results of 20 iterations of the simulation were averaged to get the final simulated data.

The experimental data can be fit with a combination of a shrinking, detaching and breaking rate, and Figure 20 proves that each of these mechanisms is relevant and plays a significant role in the degradation of molecular shuttles *in vitro*, and that their combined action (Figure 20, d) explains the observed degradation patterns.

Monte Carlo simulations relative to other experimental setups are given in the 5th part of the Appendix, and more information about the structure and the code of the Monte Carlo simulation is given in the 6th part of the Appendix.
Detachment and breaking only

(a) Detachment and breaking only

Wear and breaking only

(b) Wear and breaking only

Wear and detachment only

(c) Wear and detachment only
Wear, breaking and detachment
Figure 20. Monte-Carlo simulations. (a): Monte Carlo simulation that only take detachment and breakage into consideration, ignoring wear. (b): Monte Carlo simulation that only take wear and breakage into consideration, ignoring detachments. (c): Monte Carlo simulation that only take wear and detachment into consideration, ignoring breakage. (d): Monte Carlo simulation taking all three degradation mechanisms into consideration. The shrinking rate and breaking rate are respectively of \( k_s = 1.2 \text{ nm.s}^{-1} \) and \( k_b = 0.016 \text{ s}^{-1} \). The detachment is defined by equation (2.9), with \( a = 9.7 \times 10^{-4} \text{ s}^{-1} \) and \( b = -4.25 \). (a) to (d): Left – Evolution of the number of microtubules in the field of view over time. Right – Evolution of the length of microtubules over time. In both graphs, the orange dots represent the experimental data and the blue line results from the Monte-Carlo simulation. The experimental data are from the experiment with 1 mM ATP and a kinesin density of 500 µm².
Future directions

This research paves the way to the study of in vitro fatigue of microtubules: by analyzing the correlation that exists between the number of buckling cycles a microtubule can undergo before breaking, and the curvature of the breaking sites, we will be able to determine if and how fatigue affects microtubules. It is relevant to study the fatigue of microtubules, because fatigue could be a mechanism at the origin of microtubule breakage – it would thus be the last piece of the puzzle explaining how kinesin-powered molecular shuttles degrade in vitro.

The experiments on the study of microtubule fatigue are made possible thanks to a collaboration between the Hess Lab at Columbia University, and the Kakugo Lab at Hokkaido University in Japan. Thanks to a unique and custom-made microtubule compression chamber, the Kakugo group has demonstrated that compression stress induced mechanical deformation of microtubules, and they have investigated the role of compression strain and strain rate on the microtubule deformation. They showed that microtubules that are supported on a two-dimensional substrate undergo buckling when they are subjected to compression stress (Figure 21). The buckling was proven to strongly rely on the compression strain while the compression rate had no substantial effect on it. As of the buckling mode of the microtubules, it can be modified by using different kinesin densities on the surface.
Figure 21. Schematic and fluorescence microscopy images of microtubule buckling. The compression stress applied at the substrate caused buckling of the microtubule attached to the substrate through interaction with kinesin. Scale bar: 10 μm.

In a collaboration (not described in this thesis), we obtained preliminary results regarding this project by using the two-dimensional substrate and the protocols designed by the Kakugo group. To do so, we first anchored the microtubules on an elastic substrate, polydimethylsiloxane (PDMS), by kinesin motors. Since we did not provide any ATP to the system, the microtubules were immobilized. We then increased the constant and cyclical stress experienced by the microtubule lattice in a controlled fashion by forcing the microtubules to buckle. As a result, we were able to observe microtubules breaking due to repeated buckling: after submitting microtubules to varying compressive forces, we observed the degradation and breaking of microtubules under each condition. As expected, the higher the compressive forces, the least number of cycles were needed to induce the rupture of microtubule lattice (Table 7). Follow-up experiments and a more in-depth analysis of these results would be the next step to follow.
Table 7. Number of compression cycles needed to break microtubules under different compressive forces.

<table>
<thead>
<tr>
<th>Compression (as a percentage of the initial length)</th>
<th>10 %</th>
<th>12.5 %</th>
<th>15 %</th>
<th>20 %</th>
<th>≥ 30 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cycles to breakage</td>
<td>No breakage</td>
<td>256</td>
<td>4-8</td>
<td>1-4</td>
<td>1</td>
</tr>
</tbody>
</table>

Conclusion

Microtubules in molecular shuttles break when the kinesin motors are activated. The breakage rate is correlated with damaged sites along the microtubule or high strains induced by the buckling of the microtubule. The frequency of detachment events depends on the microtubules’ gliding velocity and the kinesin density, which is correlated with the number of defective kinesins on the surface. Thus, in order to increase the lifetime of molecular shuttles, further developments must investigate the minimization of the number defective kinesin in a batch, through modifications of the kinesin purification process and/or the use of different type of kinesin (such as kinesin expressed in insect cells).
Appendix

1. Over 90% of breaking events are caused by high curvatures

Out of the 20 breaking events presented below, the first 2 are due to background breaking, whereas the following 18 breaking events are caused by high curvatures on the microtubule.
Background breaking

(a)

High-curvature-induced breaking

(c)
Appendix Figure 1. Breaking microtubules. The first 4 images of each row follow the “mother” microtubule right before it breaks, and the last image is the first frame in which the 2 “daughter” microtubules are visible. Rows (c) to (t) show how microtubules get stuck and buckle before breaking. The white arrow on the first frame of each row points at the “mother” microtubule that breaks into 2 “daughter” microtubules in the last image of each row. The ATP concentration is of 1 mM for all pictured breaking events. The kinesin density is of 1000 µm⁻² in rows (a) to (m), of 500 µm⁻² in rows (n) to (s), and of 100 µm⁻² in row (t).
2. Frequency of curvatures

The curvature along the paths of 20 randomly selected microtubules was calculated in all experimental setups. The curvature was calculated on 1 µm segment of these paths, and the resulting distribution of curvatures are summarized in the table below.

Appendix Table 6. Distribution of curvatures along the paths of randomly selected microtubules in all experimental setups.

<table>
<thead>
<tr>
<th>Kinesin density (µm⁻²)</th>
<th>ATP concentration (mM)</th>
<th>Frequency of curvatures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>1</td>
<td>Counts</td>
</tr>
</tbody>
</table>

\[ Log_{10} (\kappa/\mu m^{-1}) \]
<table>
<thead>
<tr>
<th>Value</th>
<th>Count</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Value 1</td>
<td>Value 2</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

![Histogram](image)

Counts vs. $\log_{10}(\kappa/\mu m^{-1})$
3. Frequency of breaking curvatures

Similarly, the distribution of breaking curvature distribution of all exploitable experiments is summarized in the table below.

**Appendix Table 7. Distribution of breaking curvatures in different experimental setups.**

<table>
<thead>
<tr>
<th>Kinesin density (μm⁻²)</th>
<th>ATP concentration (mM)</th>
<th>Frequency of curvatures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Value</td>
<td>Count</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>500</td>
<td>1</td>
<td>There were only 2 breaking events in this experiment, so no relevant histograms can be plotted.</td>
</tr>
<tr>
<td>500</td>
<td>0.01</td>
<td>There were no breaking events in this experiment.</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>
4. The time to breaking is exponentially distributed

As stated in equation (3.3), the breaking can be written as:

\[ \lambda = \lambda_0 e^{\sigma / \sigma^*} \]

Considering that the breaking rate does not depend on time, the probability of a microtubule not breaking until a time \( t+dt \) can be written as:

\[ P(T > t + dt) = P(T > t + dt | T > t) \cdot P(T > t) \]  \hspace{1cm} (3.13)

where \( T \) is the time of breakage of the microtubule. So:

\[ P(T > t + dt) = \left(1 - P(T < t + dt | T > t)\right) \cdot P(T > t) = (1 - \lambda \cdot dt) \cdot P(T > t) \]

\[ P(T > t + dt) = P(T > t) - \lambda \cdot dt \cdot P(T > t) \]

So:

\[ \frac{P(T > t + dt) - P(T > t)}{dt} = -\lambda \cdot P(T > t) \]

In other words:

\[ \frac{dp}{dt} = -\lambda \cdot P \]  \hspace{1cm} (3.14)

So \( P(t) = A \cdot e^{-\lambda t} \) where \( A \) is a constant.

Knowing that \( P(0) = 1 \) because the microtubules are stabilized and do not break before the beginning of experiment, we get \( A=1 \). So, the probability of a microtubule not breaking until a time \( t \) is given by:

\[ P(t) = e^{-\lambda t} \]  \hspace{1cm} (3.15)

And the probability \( P_b \) for a microtubule breaking in a time \( t \) is given by:

\[ P_b(t) = 1 - P(t) = 1 - e^{-\lambda t} \]
5. Monte Carlo simulations of different experimental conditions

The results of the Monte Carlo simulations for the experiments with 1 mM ATP are given in the table below. The experiments with 10 µM ATP have not been included because, as described in Chapter 2, there were not enough detachment events under that condition to determine the detachment rates of the microtubules.
Appendix Table 8. Monte Carlo simulations. In the 2nd column, a
and b are the fit parameters to the equation $\log_{10}(\lambda_s) = at + b$.

<table>
<thead>
<tr>
<th>Kinesin density (μm$^{-2}$)</th>
<th>Detachment ($\lambda_d$), shrinking ($k_s$) and breaking rate ($k_b$)</th>
<th>Monte Carlo simulations</th>
</tr>
</thead>
</table>
| 1000                        | $a = 8.0 \times 10^{-4}$  
$b = -5.32$  
$k_s = 1.0 \text{ nm.s}^{-1}$  
$k_b = 0.027 \text{s}^{-1}$ | Evolution of the number of microtubules  
Evolution of the length of the microtubules |
\begin{itemize}
\item $a = 9.7 \times 10^{-4} \text{ s}^{-1}$
\item $b = -4.25$
\item $k_s = 1.2 \text{ nm s}^{-1}$
\item $k_b = 0.016 \text{ s}^{-1}$
\end{itemize}

**Evolution of the number of microtubules**

**Evolution of the length of the microtubules**
6. Code structure and MATLAB code of the Monte Carlo simulation

The structure of the MATLAB Monte Carlo simulation is given below, followed by the code of the different functions programmed.
Appendix Figure 2. Flow chart of the code of the Monte Carlo simulation.
Each box of the in Appendix Figure 2 represents a different step in the Monte Carlo simulation. First, the user manually loads the initial length and number of microtubules of the simulation based on experimental data. The user also chooses the shrinking rates, the breaking, detaching and sticking probabilities to be used in the simulations. Then, the simulation successively immobilized, shrinks, breaks and detaches microtubules following the entered rates. Certain immobilized microtubules start moving again, and certain others get immobilized. The number and length of microtubules for that time step is saved in a matrix and the time is incremented. Using the new population of microtubules, the simulation goes through the shrinking, breaking, detaching and sticking/unsticking steps again until a time of 1800 s is reached, at which point the evolution of the length and number of microtubules is recorded. At that point, the initial experimental population of microtubules is set again as a starting point and the whole process repeats itself again, for a total of 20 times. At the end of the 20 iterations, the number and lengths of microtubules at each step is average and provided as the output of the simulation.
Main function: “Monte_Carlo.m”

```matlab
function [Nb_MTs, av_length_simu, t, final_dist] = Monte_Carlo(iniDist, max_t, t_step, l_rate, l_const, s_rate, b_rate)

% iniDist = .mat file that contains initial distribution variable
load(iniDist, 'initial_distribution');

loops = 10; % number of loops of the simulation
max_time = max_t*60; % convert max time to seconds
time_step = t_step; % time step
t=0:time_step/60:max_time/60; % distribution of time steps for sim
min_length = 0.05; % for microtubules (in μm)

% Matrix that will hold the number of microtubules at every time step
Nb_MTs = zeros((max_time/time_step)+1,loops);
% Matrix that will hold average length of the microtubules at every time step
av_length_simu = zeros((max_time/time_step)+1,loops);
% Saving the distribution of lengths at end of the simulation time
final_distribution = cell(1,loops);

leaving_rate= l_rate; % a parameter of the exponential function giving the detachment rate
leaving_constant= l_const; % b parameter of the exponential function giving the detachment rate
shrinking_rate = s_rate; % μm/60s
breaking_proba = b_rate; % probability that a microtubule will break in 1 min
stick_rate = 0.005; % probability that a microtubule will get immobilized in 1 min

for i=1:loops

% all runs have the same starting point
MT_sim=initial_distribution';
time = time_step;
Nb_MTs(1,i) = length(initial_distribution) ;
av_length_simu(1,i) = mean(initial_distribution) ;
b=1;

% Create a starting set of microtubules that are stuck to the surface
% The set needs to have a certain number of microtubules below a certain length
% since the immobilized microtubules are usually the small broken
% ends of microtubules that broke
MT_immob = [];
numImmobilized = 40; % 20 for 1mMk10, 40 for 1mMk20, 5 for 1mMk100
maxLengthImmob = 2; % 1.5 for 1mMk10, 2 for 1mMk20, 10 for 1mMk100
count=0;
while(length(MT_immob)<numImmobilized)
    if(length(MT_sim)<1)
        break
    end

    idx = randi(length(MT_sim));

    if(MT_sim(idx)<maxLengthImmob)
        MT_immob = [MT_immob MT_sim(idx)];
        MT_sim(idx) = [];
    end

    count=count+1;
    if(count>200)
        break;
    end
end
```

while isempty(MT_sim)==0 && time<=max_time

    % Setting the photobleaching rates of the microtubules
    % bleach_rate = 0.336*exp(-0.106*time/60); % lmK10
    bleach_rate = 0.0542*exp(-0.033*time/60); % lmK20
    % bleach_rate = 0; % lmK100

    % Wear
    MT_sim = Wear(MT_sim, shrinking_rate*time_step/60); % Shrinking
    MT_immob = Wear(MT_immob, bleach_rate*time_step/60); % Photobleaching
    mts_broken = [];

    % Breaking
    for q=1:length(MT_sim)
        mts_broken = [mts_broken Breaking(MT_sim(q), breaking_proba*time_step/60)];
    end
    MT_sim=mts_broken;

    % Remove the microtubules those length is below the minimum length
    [MT_sim2] = MT_sim(MT_sim>min_length);
    [MT_immob2] = MT_immob(MT_immob>min_length);
    MT_sim=MT_sim2;
    MT_immob=MT_immob2;

    % Leaving
    uleave=1/(leaving_constant*leaving_rate)*expnd(leaving_rate*time,1,length(MT));
    [MT_sim3] = MT_sim(uleave=(leaving_rate*time_step/60));
    MT_sim=MT_sim3;

    % Sticking
    [MT_sim,MT_immob] = Sticking(MT_sim, MT_immob, stick_rate, maxLengthImmob);

    % Go to the next iteration
    k=k+1;
    Nb_MTs(k,i) = length([MT_sim MT_immob]);
    av_length_simu(k,i) = mean([MT_sim MT_immob]);
    time = time + time_step;
    if(time > max_time)
        final_distribution(1,i) = [MT_sim MT_immob];
    end
end

dbstop if error

% Return the average of all lengths and numbers
% of all loops at each time step
Nb_MTs = mean(Nb_MTs,2);
av_length_simu = mean(av_length_simu,2);
final_dist = final_distribution;
t = t';

Function: “Wear.m”

function [MT] = Wear(MT, shrinking_rate)
    MT = MT - expnd(shrinking_rate,1,length(MT));
end
**Function: “Breaking.m”**

```matlab
function [MTs] = Breaking(MT, breaking_proba)

if (rand()) <= breaking_proba
    p = exprnd(1);
    % The microtubules will break with an exponential profile around 1 μm
    % from their leading end
    if p > MT
        MT1 = MT/2;
        MT2 = MT - MT1;
        MTs = [MT1, MT2];
    else
        MT1 = p;
        MT2 = MT - MT1;
        MTs = [MT1, MT2];
    end
else
    MTs = [MT];
end
```

**Function: “Sticking.m”**

```matlab
function [MT_sim, MT_immob] = Sticking(MT_sim, MT_immob, stick_rate, maxLengthImmob)

    % Split the microtubules into sets of microtubules small enough
    % to stick
    MT_sim4 = MT_sim(MT_sim<maxLengthImmob);
    MT_sim5 = MT_sim(MT_sim>=maxLengthImmob);

    % Choose the microtubules based on rate based on number of microtubules
    % that are small enough to stick
    ustick = unifrnd(0, 1, 1, length(MT_sim4));
    [stuck_MTs] = MT_sim4(ustick<(stick_rate*length(MT_sim)/length(MT_sim4)));
    [unstuck_MTs] = MT_sim4(ustick>(stick_rate*length(MT_sim)/length(MT_sim4)));
    [MT_sim] = [MT_sim5 unstuck_MTs]; % return unstuck microtubules to the original set
    MT_immob = [MT_immob stuck_MTs]; % add new stuck microtubules to the immobilized set
end
```
Chapter 4.

Case-based Teaching in a High-Level Engineering Course Enhances Students’ Participation and Learning

Introduction

In the past two decades, extensive research on teaching methods has provided an increasing amount of insight in the most effective active teaching and learning processes. The outcomes of different studies vary, but a major consensus is that active learning works. It has been proven that at worst, active learning doesn’t harm students’ outcomes; but at best, it doubles students’ retention. Consequently, active learning was incorporated in fields such as Science, Technology, Engineering and Mathematics (STEM), history and political sciences, and business. Each field, however, focused on different active elements: business school courses are traditionally known as mostly case-study based, while engineering courses traditionally focus more on lectures and problem-solving exercises. However, a combination of both approaches in the same course has not yet been described. We tackled this challenge in our 4000-level course “Fundamentals of Nanobiotechnology and Nanobioscience”.

When designing the course, we relied on a framework that helped in the conception of relevant, engaging, and practical classes. The “backward design” approach by Wiggins and
McTighe\textsuperscript{122} fulfills these criteria. This method first raises questions such as: “what should the students learn from this course?” and “what skills should the students acquire by the end of the semester?” instead of teaching a class by following chapters from a textbook. By addressing these questions, the instructor can set the objectives of the course before creating their lesson plan. Rather than using pre-established structures for their course, they can determine the teaching elements that would be the most appropriate to reach those objectives. By continuing to move “backwards”, the topics to be emphasized can be selected, and lastly, the content of each class can be set.

Here, using “backwards design”, we adopted a hybrid approach, and presented a multi-leveled active course. We created a new course structure that incorporated active elements that are traditionally used in business school curricula in an engineering course. By doing so, the “Fundamentals of Nanobiotechnology and Nanobioscience” course aims to get students more involved and engaged in the learning process, which in turns will improve both their classroom experience and their retention of the material.

Methods

Setting the course’s learning objectives

Using “backwards design”,\textsuperscript{122} we started by defining the learning objectives we had for the students. We targeted several levels of learning as described by Bloom’s taxonomy,\textsuperscript{123,124} which spanned from the lowest level – which consists of recalling facts and concepts – to the highest one – being able to produce a new or original idea or work in the area. At the end of the course, we wanted for the students to be:

1. Able to define nanobiotechnology in the context of modern science and engineering,
2. Capable of understanding and interpreting concepts such as intermolecular bonds, adsorption and binding/unbinding processes, nanoscale transport mechanisms, and degradation mechanisms at the nanoscale,

3. Comfortable in estimating orders of magnitude of objects that relate to engineering,

4. Capable of comparing and evaluating research papers related to nanobiotechnology with a critical mind,

5. Able to take a position towards an engineering-related question and defend their position in front of others,

6. Able to describe examples of applications and outline the state of the art in nanobiotechnology,

7. Able to contribute to and build upon team ideas through discussion.

Designing the course

Once the learning objectives were set, the structure of the class was considered as a means to reach these objectives. Relying on the positive effects of active learning, we designed a novel course format in which the class-time was divided around three different types of activities that succeeded each other using the pattern presented in Figure 22. The topic of each unit reflected a key area that we aimed to investigate in the course, and we focused on topics that are both more favorable to generate longer class discussions, and who have been researched in the past. Examples of such topics include: “Nanobiotechnology and its applications”, “Lifetime of nanobiodevices” or “Reproducing macroscale bonds at the nanoscale”.
Figure 22. Basic unit of the “Fundamentals of Nanobiotechnology” course. Each “cycle” focuses on one key topic related to nanobiotechnology. It consists of one or two lectures on a topic, followed by a case study and two case histories – adding up to a total of four or five sessions dedicated to this topic. The course was composed of five cycles of the type.

Lectures

Lectures were the most traditional elements of engineering teaching that were implemented in this course. They made up less than half of the overall class time, and they provided the students with enough background material to be able to address the questions that were raised in the following case study and case histories. Lectures, however, were not passive.

First, lectures were partially “flipped”: the most important and foundational material of each lecture was recorded and divided into one or several short videos. The students had to watch the videos in preparation for the class. To ensure their engagement in this process, after watching the video, the student had to take a short online quiz. These short quizzes emphasized the main
take-away points of the online lecture. Once in class, the instructor drew the students’ attention on the main take-home messages of the flipped lecture, explained the topics that were less understood by a majority of students, and the students had the opportunity to ask for clarifications. The links to the Youtube videos of the flipped lectures is given in the Appendix.

Furthermore, flipped lectures opened up time for making the in-class lectures more active. All lectures incorporated elements for active learning such as think-pair-share exercises\textsuperscript{125} or small group discussions, the overarching aim being to bring the students not only to a higher level of understanding, but also to teach them to develop, formulate and justify their ideas.

**Case Studies**

Collaborative learning improves learning outcomes in a broad range of aspects from academic achievement\textsuperscript{126,127} to students’ retention of the material\textsuperscript{128,129}. Collaborative learning was introduced in this course through the use of business-school inspired classes. In groups of 3 or 4, students had to think about research questions related to the previous lectures’ material. The groups were chosen based on the students’ affinities, and the instructor encouraged the students to work with different people throughout the semester. The basic structure of case study classes (Figure 23) would be repeated 3 or 4 times during the 75-minute class. All questions were broad and open-ended, but got gradually more specific and thought-provoking. For instance, the first question of the case study was often about the most interesting research direction to follow based on the material that was taught in the previous lecture. The next question investigated the feasibility or the implementation of the research ideas the groups came up with; and the final question would dive into the in-depth analysis of the economical or societal pay-off of that research. For instance, the questions that were asked during the “Reproducing macroscale bonds at the nanoscale” were:
1. How can you create a bond at the macroscale? Name and describe as many bond-creating mechanisms as you can.

2. How can you create a bond at the nanoscale? To what macroscale mechanism(s) are they similar?

3. If you had to reproduce a macroscale bond (that you haven’t cited in the previous question) at the nanoscale, which one would it be and how would you do it?

![Image of a case study session pattern](Figure 23. Pattern of a case study session. This pattern was repeated three or four times per case study, depending on the difficulty of the questions and the students’ participation.)

Meanwhile, the instructor circulated between the groups, aligned the students’ perspectives towards the learning objectives of the session, and generated longer class discussions. These classes exclusively consisted of discussions about the real-life applications of the material, so they automatically turned the classroom into a more active and interactive environment in which the teaching was much more personalized.

Furthermore, the first case study, entitled “What are the future prospects in nanobiotechnology?” was repeated as the last case study of the course. We recorded the responses
given by the different groups the first time they were presented with the topics, and presented the students with their own answers the second time. Thus, through the critique of their own answers, the students were able to visualize their own progress.

Case histories

The case histories consisted in reading, understanding and critiquing of papers that could be considered as responses to the previous case study. They presented what research has been done in relation to the preceding lecture material and case study. By showing how the course’s material is currently used and looked upon by researchers, the case histories were intended to give the students an idea of all the different ramifications of the field of nanobiotechnology. Thus, the case histories served as conclusions to the three or four classes spent on a specific subject. Typical questions that were asked during case history sessions include:

1. What was the state-of-the-art in the field before this paper was published?
2. Why is the research presented in this paper relevant to nanobiotechnology?
3. Critique the paper: if you had to write it, what would you keep, and what would you do differently?

In a few cases, we also interviewed the first author of a case history paper. The interviews addressed two major topics: the paper itself, and the author’s professional career. First, the history behind the scientific article was investigated through questions about the time it took to get results, the publishing process, the easiest or hardest part of the experiments, etc. Then, to expose students to different career paths that exist in nanobiotechnology, the authors were questioned about their career and its evolution since they finished their graduate studies.
Thanks to this course format, the students were active in learning the course material before, during and after the time allocated to the class (Table 8).

Table 8. Students’ involvement in the course throughout a course “unit”.

<table>
<thead>
<tr>
<th></th>
<th>BEFORE THE CLASS</th>
<th>DURING THE CLASS</th>
<th>AFTER THE CLASS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LECTURES</strong></td>
<td>Watch lecture videos</td>
<td>Participate in discussions</td>
<td>Prepare for the next class</td>
</tr>
<tr>
<td></td>
<td>Answer to online quizzes</td>
<td>Ask for clarifications</td>
<td>Do the assigned homework</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Defend one’s ideas</td>
<td></td>
</tr>
<tr>
<td><strong>CASE STUDIES</strong></td>
<td>Review lecture material</td>
<td>Participate in discussions</td>
<td>Prepare for the next class</td>
</tr>
<tr>
<td></td>
<td>Do the assigned homework</td>
<td>Work on the case with one’s team</td>
<td>Do the assigned homework</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Present one’s team’s answers to the questions</td>
<td></td>
</tr>
<tr>
<td><strong>CASE HISTORIES</strong></td>
<td>Review lecture material</td>
<td>Participate in discussions</td>
<td>Prepare for the next class</td>
</tr>
<tr>
<td></td>
<td>Review case study</td>
<td>Be active in group activities</td>
<td>Do homework</td>
</tr>
<tr>
<td></td>
<td>Read case history paper</td>
<td>Critique the paper</td>
<td>Watch online interviews</td>
</tr>
</tbody>
</table>

Design of the classes

Once the format of the course was defined, each class was conceived with the aim of addressing one or more of the previously cited learning objectives. Suitable examples and activities were determined and incorporated in them.

Teaching the class

The course “Fundamentals of Nanobiotechnology and Nanobioscience” has been taught as an integral part of the semester course schedule in the School of Engineering and Applied Sciences
of Columbia University since Spring 2017. It is intended for senior undergraduate students as well as graduate students. The class consists of 28 75-minute long lectures, as well as a 3-hour long final. In Spring 2017, 7 students enrolled in the course, 17 students enrolled in Spring 2018, and in Spring 2019, this number rose to 27 students. The students were from different academic backgrounds: they majored in fields such as Biomedical engineering, Biotechnology, and Mechanical Engineering.

Assessment of the course

Quizzes

The online quizzes that accompanied the flipped lectures acted as a continuous formative assessment tool for the instructors. By analyzing the outcome of the quizzes before the beginning of the next class, the instructors were able to implement methods of Just-in-Time Teaching (JiTT) by adjusting the material to the students' needs. For instance, if a specific concept was misunderstood by the students, the instructors became aware of it thanks to the quizzes. As a result, they would dedicate a few minutes of the next class to explain that concept and/or add details about it in the lecture video that would be used in the following years.

To encourage the students to engage with the material, 10% of the course's grade was contingent upon their taking the quizzes.

Homework

Homework sets were intermediaries between summative and formative assessments. Five homework sets were designed using backwards design. Each set addressed at least two of the learning objectives of the course, and a special emphasis was put on the course’s third learning
objective (i.e. being comfortable in estimating orders of magnitude of objects that relate to engineering). Each assignment grade accounted for 20% of the total homework grade, and the average score of the homework sets counted for 40% of the course grade.

Final

The final exam of the course was the most prominent summative assessment of the students’ learning. Once again, each question was designed by referring to the learning objectives of the course. The final exam was 3 hours long, closed book, and tested the students’ knowledge of the key concepts addressed in the course. It accounted for 50% of the course grade.

In order not to penalize the more introverted students, student participation was not graded as part of the course, but counted as extra credit: students could get up to 3% extra credit based on their participation.

Assessment of the active elements

Pre-course and end of course surveys

The validity and reliability of self-reported data has been discussed for nearly half a century.\textsuperscript{130-132} Now, researchers generally agree that self-reported estimates of learning are valid,\textsuperscript{133-135} without necessarily being a substitute for objective measures\textsuperscript{136}. For instance, Anaya et. al determined that GPA, student-reported growth, and scores on the GRE are all three valid measures of learning.\textsuperscript{131} Relying on these findings, we designed two optional and anonymous surveys, one of which was taken during the first week of the semester (this survey will later on be referred to as the pre-course survey), and the other one was an end of course survey. The students’
self-reported responses were then analyzed in order to assess the effectiveness of the implemented active measures.

The pre-course survey consisted of two questions aimed at gauging the students’ knowledge on both the material that would be taught in the course, and the non-topic-specific learning objectives of the course. The post-course survey consisted of the same questions as the pre-course survey, and also included (a) an additional multiple-choice question regarding the progression of the course and (b) four open-ended questions about the different active components of the course, as well as an evaluation of overall strengths and weaknesses of the course. The survey questions and the students’ answers are provided in Figures 23 to 26.

**Student participation**

Each student’s participation was evaluated throughout the semester. This evaluation, which started after the drop period for the semester ended (for consistency), recorded the number of times each student participated in the class, whether by asking a question, expressing an idea, or reporting a group’s response to an in-class exercise. One-word answers – such as “yes” or “no” – or non-course specific question – such as asking to rewrite a word on the board for readability purposes – were not taken into account.

**Results**

**End of course survey**

Results from the end of course surveys are presented in Figures 23 and 24. This optional end of course survey was completed by 7 out of 7 students in Spring 2017, and by 10 out of 17
students in Spring 2018. The survey questions and course content having remained the same for both semesters, the cumulative results from both semesters are presented here.

Figure 24. Students’ end of course self-evaluation of the extent to which the learning objectives of the course had been met. The x-axis represents the percentage of students falling within each category.
Figure 25. Students’ end of course evaluation of the active tools used during the course. The x-axis represents the percentage of students falling within each category.

Comparison of pre- and post-course survey data

Anonymous pre-course survey results were collected in Spring 2018, and 14 out of 17 students responded to it, while 10 out of 17 students responded to the end of course survey. The histograms presented in Figure 26 are derived from the comparison of the pre-course survey and the end of course survey responses regarding the course’s learning objectives. As of the questions addressed in Figure 27, they assess the broader impact the course had on teaching students to think both scientifically and critically.
(a) I am able to define nanobiotechnology in the context of modern science and engineering

- Strongly Agree
  - End of course survey
  - Pre-course survey

- Agree
  - End of course survey
  - Pre-course survey

- Neither agree nor disagree
  - End of course survey
  - Pre-course survey

- Disagree
  - End of course survey
  - Pre-course survey

- Strongly disagree
  - End of course survey
  - Pre-course survey

(b) I am able to describe examples of applications and outline the state-of-the-art in nanobiotechnology

- Strongly Agree
  - End of course survey
  - Pre-course survey

- Agree
  - End of course survey
  - Pre-course survey

- Neither agree nor disagree
  - End of course survey
  - Pre-course survey

- Disagree
  - End of course survey
  - Pre-course survey

- Strongly disagree
  - End of course survey
  - Pre-course survey
Figure 26. Comparison of the pre-course and end of course survey for questions regarding the course’s learning objectives.

The x-axis represents the percentage of students falling within each category. (a) The question asked is a rephrased version of the first learning objective of the course. This learning objective tackles the
lowest level of Bloom’s taxonomy, recalling facts and basic concepts. (b) The question asked is a rephrased version of the sixth learning objective of the course. This learning objective tackles the second lowest level of Bloom’s taxonomy, explaining and describing ideas and concepts. (c) The question asked is a rephrased version of the third learning objective of the course. This learning objective tackles the second highest level of Bloom’s taxonomy, evaluating and justifying a stand or a decision. (d) The question asked here is a rephrased version of the seventh learning objective of the course. This learning objective tackles the highest level of Bloom’s taxonomy, designing new or original works and responses.
(a) I can identify the goals and objectives of a paper

Strongly Agree
Agree
Neither agree nor disagree
Disagree
Strongly disagree

(b) I can point out the strengths and weaknesses of a scientific paper

Strongly Agree
Agree
Neither agree nor disagree
Disagree
Strongly disagree
Figure 27. Comparison of the pre-course and end of course survey for questions regarding the broader impact of the course on the students’ approach to research and engineering. The x-
axis represents the percentage of students falling within each category.

Written Feedback

At the end of course survey, students had the option to express their opinion on the course’s strengths and weaknesses. All the students who took the survey also gave feedback on the course.

Representative student comments are reproduced below.

Comments on the course in general:

*Overall, this was my favorite class this semester, I very much enjoyed the homework assignments that elicited both a creative thought process and problem-solving skills.*

*Wonderful class and instruction. Course made me think critically and made me feel like I was learning things in a purposeful way, rather than just for the sake of learning.*

*Makes you think about problems conceptually and deeply in order to allow students a fuller understanding. Course structured in a way that allows knowledge to be built upon class after class.*

*I truly enjoyed this class and learned so much from it.*
Comments on the biggest strength of the course:

Course structure, online assignments and resources, lecture style that encourages student engagement.

I would have kept the number and format/style of homework and quizzes the same.
I liked the in-class lectures they were solid.

I would not have changed a thing.

Comments on possible improvements to the course:

I personally would have been interested in learning a few more applications of nanobiotechnology in medicine.

It might have been nice if the case histories emphasized a broader range of topics in nanobiotechnology than motor proteins.

Solidify (lecture) notes and have them posted after the lecture.

I would have tried to keep students updated with materials from the lectures by posting notes online. Also, I would have tried to communicate more with students in terms of the progress of the course (as in reminding what is due when, for example) through email.

Maybe set reminders for coursework to email?
A lot of ideation came from the classroom, but was unsure if we covered every possible or major idea that was related to a topic (I'm assuming we did) but starting us off in appropriate directions would have maybe been more interesting to prompt discussion.

Comments on flipped lectures:

Thoroughly enjoyed them.

I am personally a big fan of the flipped lecture style and thought it helped with my comprehension of the papers we read in advance.

Flipped lectures are great but it wasn't always timed or content matched exactly.

Keep doing them.

Comments on case studies and case histories:

While it was definitely something we discussed in nearly every case study, I think addressing the "state of the art" at the time of each paper's publishing is especially important to provide context and emphasize progress.

I like the case studies yes. It would be nice if this class was taught in a more collaborative classroom that facilitates more meaningful conversations.
I thought that case histories are insightful and I liked how you always placed case histories after case studies, since case study portrays a problem and case history describes a solution. It was fun to see what the scientists actually came up with as solutions to the problems.

A potentially good application of all the case studies would have been a final project oriented around theoretically designing something that build off all the material rather than just an exam.

Perhaps providing the class with some preparatory questions or topics to research could be beneficial and move things along even more during the short class times.

[They] helped better understand underlying concepts.

Comments on the last case study of the course:

I think reviewing the discussions from the very beginning of the semester regarding the potential applications of the nanopropeller was a valuable exercise. Distinguishing the lofty, long-term goals of nanotechnology from the accomplishments of individual papers is an important way to illustrate the difference between 'goal' and 'objective'.

It showed how much we learned and that was cool.
I have a more realistic and thoughtful process of evaluation when reading it the second time around.

Yes, it was informative to discuss the same case study with a more extensive understanding of the current "state of the art" in nanobiotechnology.

I think this is just to complete the circle. By coming back to what you started out with at the end, you can see how everything ties in with each other in the course.

Student participation

The student-specific and class-specific participation data for Spring 2018 are represented in Figures 28 and 29. The average student participation in the different types of classes (lectures, case studies, and case histories) is shown in Figure 30.
Figure 28. Students’ active participation throughout the semester. Each number on the x-axis represents a student, while each number on the y-axis represents the number of times that student voluntarily participated in the course.
Figure 29. Average number of participations per student in each class. The total number of student participations in a class was divided by the number of students present in that class in order to get the above data points. There is no data for the period between March 12th and March 16th due to spring break. Due to high variations between individual students’ participation, error bars have not been added to this graph.
Figure 30. Average number of times a student participated in each class of each type. The error bars represent the standard error of the mean. Student t-tests show that the difference between participation in lectures and the other two types of classes is statistically significant at the 5% level, whereas the difference between the case studies and the case histories is not statistically significant.

Discussion

Survey data

By getting students more involved and engaged in the learning process, we aimed to address a broader range of learning objectives, and the students believed that all learning objectives
have been met (Figure 24). Since every student has their own learning preferences, there are a few people who do not agree that all the active elements of the course were as useful, but overall, students claim that the course structure encourages active learning and gives a more applied perspective on engineering; for them, the active elements were useful, and they got more comfortable answering the broad case study questions as the semester went on (Figure 25).

Figure 26 highlights the fact that the students feel that they progressed in the acquisition of the set of skills defined by the learning objectives were aiming at, independent of the level of those skills in Bloom’s taxonomy. This is all the more relevant for the learning objectives that tackle the higher levels of learning since they do not only concern nanobiotechnology per se: rather, they concern engineering in a more general sense and their goal is to teach students to have the proper mindset when confronted with engineering-related problems. This is corroborated by the results presented in Figure 27: even though this course was not a Journal Club, students were more comfortable in reading, understanding and critiquing a journal publication at the end of the semester.

Student feedback

The students written feedback informs us that overall, the students appreciated the structure of the course as well as its assessment methods. They enjoyed the course and were aware of their own progress. When asked about the possible improvements that can be made to the course, three themes were recurrent:

1. Being sent email reminders concerning the coursework deadlines.
2. In addition to lecture videos, posting the instructor’s lecture notes online.
3. Being presented with a broader range of applications.
This feedback is very valuable, since it informs on how to improve the course at successive iterations.

**Student participation**

The student-specific participation data (Figure 28) helps visualize the types of classroom behavior students have: about one half of the students are overall less inclined to participate in in-class discussion (data points 1 to 8), while we can observe an increasing range of participation for the other half of the students (data points 9 to 17). Even though we can consider the second half of students as active and engaged with the course material, these results do not imply that the less participative students were not engaged in the course. The observed differences in student participation can be attributed to a number of factors, including the students’ personalities, their background and their interest in the course.

As expected, students were more active in case study and case history classes, which were designed as more active classes (Figure 30). It is also interesting to note that, excluding the participation on the March 8th class, the students’ participation is U-shaped (Figure 29): the participation is high at the beginning and the end of the semester, and is at its lowest mid-semester. This can be a reflection of students’ motivation and/or morale, since the relationship between student morale and/or expression of depressive symptoms was proven to be highly correlated with midterms and major assignments. More specifically, students’ morale sharply plummets around the time of midterms, which has even been referred to as the “Midterm Blues”. Here, we prove that there is a correlation between student participation and morale, regardless of the students’ more introverted or extroverted personalities. This can in itself be the subject of future investigations.
Lastly, as of the outlier represented by the March 8th, it is interesting to note that that class took place only a couple of hours before the students went on Spring Break, in other words at a time when all of their midterms had just ended. Once again, this supports the idea that higher morale is correlated with more active participation.

Conclusion and future directions

The “Fundamentals of Nanobiotechnology and Nanobioscience” course is now a well-established course in Columbia University’s Biomedical Engineering department. Student self-report that they appreciate the course and the efforts made to make it more active. While the obtained results are positive and encouraging, we aim to continue to improve the course in the future by taking the students’ feedback into account. For instance, we posted the lecture notes online and addressed a broader range of applications in the 2019 iteration of the course. Furthermore, we hope to apply this structure to other courses in biomedical engineering, thus enhancing the students’ experience, their active interaction with the material, and as a result, their learning.

Lastly, another future research direction that we can take is to study the effect of the “Midterm Blues” more in details. Our research highlighted the correlation between student’s ease and happiness of mind and their in-class participation. By expanding this study to different classes, and by quantifying the students’ proportion to do sports or have social interactions throughout the semester, one can quantify how deep and widespread this “blues” is. As a result, this would provide us with information regarding the students’ mental health and thus the instructors would be able to better adapt their teaching to the students’ psychological needs as well.
Appendix

1. Flipped lectures

The flipped lectures can be found at the following links:

Lecture video 1: Kinesin motor proteins

https://youtu.be/2FSIJ0CyOEE

Lecture video 2: Myosin motor protein

https://youtu.be/LGohoL3T UWQ

Lecture video 3: Diffusion

https://youtu.be/3vyuwzR48QpE

Lecture video 4: Other transport mechanisms

https://youtu.be/s1QFWsc11PQ

Lecture video 5: Bond rupture

https://youtu.be/R1aeM4t63hs

Lecture video 6: Persistence length

https://youtu.be/ikgtpxzKjQc

Lecture video 7: Friction and wear

https://youtu.be/JKSMo_Zv6R8
Chapter 5.

Conclusion

Understanding the degradation of \textit{in vitro} molecular shuttles

Polymerized microtubules remain intact for about a week when stabilized with paclitaxel and kept in a buffered solution, and for several hours when in presence of inactive kinesin motors. However, their stability is significantly reduced when the microtubules are in the presence of active kinesin motors. More specifically, the degradation of microtubules was characterized as a function of kinesin motor density and time, and 3 mechanisms combine to shorten the lifetime of the system:

1. Microtubules experience wear

Previous works have studied the shrinking of microtubules due to wear.\cite{3,75}

2. Microtubules detach from the surface

Microtubule detachment is a non-negligible phenomenon in inverted motility assays that was surprisingly not looked upon until now. Microtubule detachment events depends on the kinesin density and the microtubules’ gliding velocity, and the detachment rate significantly increases with time, especially in assays with higher microtubule velocity. Microtubule detachment limits the average gliding distances before detachment to a few millimeters, that is to
say to many orders of magnitude less than expected based on theoretical predictions. These findings have to be taken into account for the design of future nanodevices incorporating cytoskeletal filaments propelled by surface-adhered biomolecular motors. Further investigations could study how differently-labelled microtubules and differently-expressed kinesin motors would affect the detachment patterns observed here.

3. Microtubules break

For the first time, the breaking of microtubules was studied in molecular shuttles. The breakage of microtubules in vitro was shown to be predominantly correlated with high strains experienced by the microtubule lattice. In order to reduce the number of breaking events and increase the lifetime of molecular shuttles, further developments could investigate the minimization of the number defective kinesin in a batch, and/or the use of different type of kinesin (such as kinesin expressed in insect cells).

This research also paves the way to the study of in vitro fatigue of microtubules: the fatigue of protein assemblies has not yet been vigorously studied at the nanoscale because it is challenging to apply controlled, subcritical forces on the order of a few piconewtons to supramolecular assemblies at that scale. In our experimental setup, at each step they take on microtubules, the kinesin motors exert a repetitive, subcritical force on the associated microtubule, potentially causing the system to “fatigue”. By following microtubules until they break, one could investigate how fatigue happens at the micro- and nanoscale, and how that compares to fatigue at the macroscale.
The three above-mentioned mechanisms put together fully explain the evolution of both the number and the length of microtubules that are observed in the field of view, thus giving us a clear idea of all the mechanisms involved in the *in vitro* degradation of molecular shuttles, as well as the extent of the role each mechanism plays. With the contributions made in this dissertation, the limits of the lifetime of molecular shuttles have been defined with much more precision than ever before, making it possible to better choose the experimental conditions of future studies of molecular shuttles.

**Designing a hybrid engineering course that enhances the students’ participation and learning**

We designed and taught 4 iterations of the “Fundamentals of Nanobiotechnology and Nanobioscience” course. The results were positive and encouraging: the active classes indeed increased student participation, and the students’ self-reported assessments showed that they enjoyed learning in that environment and that all the course’s learning objectives were tackled, including those who aimed at higher levels of learning according to Bloom’s taxonomy. Further developments on the subject could take 3 directions: (1) Continuing to improve the course by taking into account the students’ feedback and quantifying the added-value of the improvements; (2) Applying this class structure to other engineering courses; and (3) Studying the effect of student morale more in depth, as we demonstrated that there was a clear correlation between the “Midterm Blues” and the students’ engagement and participation in the class.
References


61. Schmidt, C.; Vogel, V., Molecular shuttles powered by motor proteins: loading and unloading stations for nanocargo integrated into one device. Lab on a Chip 2010, 10 (17), 2195-2198.


Appendix

Publications and Presentations

Peer-reviewed publications


• **Bassir Kazeruni, N. M.** and Hess, H. “Case-based Teaching in a High-Level Engineering Course Enhances Students’ Participation and Learning”, *Proceedings of the 2019 ASEE Annual Conference & Exposition*, (2019) – Accepted, to be published.


**Oral presentations**

• **Bassir Kazeruni, N. M.,** and Hess, H. "Understanding the biological and mechanical properties of molecular shuttles", *Guest speaker* at Kyoto University, Japan (January 2019).


• Panelist on a discussion on “Producing Effective Learning Materials to Support Instruction” at the *2017 Celebration of Teaching and Learning Symposium* at Columbia University (March 2017).

**Poster presentations**


• **Bassir Kazeruni, N. M.** and Hess, H. “Designing an Engineering Course Combining Case-based and Lecture-based Teaching.” *2018 Celebration of Teaching and Learning Symposium* at Columbia University (March 2018).

• Carriero, A., **Bassir Kazeruni, N. M.**, et al. “Age and Gender Influence Tissue Strains in Mouse Tibia.” *ORS 2014 Annual Meeting* (July 2014).

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• *International Fellowship for Research in Japan* by the Japan Society for the Promotion of Science (JSPS), 2019.

• *Hybrid Learning Course Redesign and Delivery* grant from Columbia University’s Office of the Provost, 2016.