INTRANEURONAL TRANSPORT IN VITRO: DEVELOPMENT OF A HIGHLY SENSITIVE MICROTUBULE BASED ASSAY

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ABSTRACT

Microtubule (MT)-based intraneuronal transport is often altered in several neurological diseases including neurodegeneration. To understand complex molecular processes underlying MT-based neuronal transport, e.g. stabilization of MT by its associated proteins, a reliable and sensitive in vitro assay is required. Such assay would permit analysis of various conditions and components affecting MT-based transport, facilitating identification of drugs modulating disease course, and ultimately resulting in the development of new theraupetic approaches. This paper focuses on improving the protein-MT interaction assay based on the reconstructed kinesin/microtubule system. Different MT immobilization processes were compared on tau protein detection. The system using MTs suspended between microstructures provided higher statistical significance.

KEYWORDS

Microtubule, Kinesin, Tau, Neuronal transport.

INTRODUCTION

Several types of neurodegenerative diseases, e.g. Alzheimer`s disease (AD), are caused by the intraneuronal transport deficiencies. [1] Therefore, understanding the analysis of intraneuronal transport and subsequent identification of agents modulating this process are in high demand. Microtubules are essential components of neurons and thus, MT-based transport have to be examined to identify the factors affecting intraneuronal transport. As a result, a reliable, sensitive and rapid analysis system investigating the components and conditions affecting intraneuronal MT-based transport would be crucial.

Microtubules, one of the main components of the cytoskeleton, are essential for intracellular transport. They have polymeric structures of 13 protofilaments, assembled from tubulin monomers. Kinesin, a linear motor protein, performs unidirectional motion along MTs by hydrolyzing ATP and carries cargo such as a vesicle containing neurotransmitters. *In vitro* studies showed that kinesin velocity is affected by the existence of molecular attachment on MTs. [2]

In this work, we investigated the effect of immobilization techniques of MTs on kinesin-coated bead motility. We propose an alternative geometry for MT immobilization, i.e. suspending MTs between two micromachined walls, to the conventional attachment on a glass surface. The sensitivity performances of different immobilization techniques of MTs were measured in a protein detection system. The results show that the proposed bioassay provides higher significance of sensitivity in comparison to conventional attached-MT-based bioassays. [3]

EXPERIMENT

Molecular Preparation

Tubulin (4 mg/ml) was polymerized into MTs in BRB80 buffer (80 mM PIPES-NaOH pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) containing 1 mM $MgSO_4$ and 1 mM GTP. After incubating at 37°C for 30 min, MTs were diluted 100-fold and stabilized with 20 μM paclitaxel.

Biotinylated kinesin was used to carry 0.49 μm diameter streptavidin coated polystyrene beads (Bangs Labs). Biotinylated kinesin (final concentration of 0.01 mg/ml) was attached on streptavidin-coated beads (final concentration of $6x10^8$ bead/ml) via specific biotin-streptavidin binding. For MT immobilization, wild type inactive kinesin was used.

For protein detection experiments, microtubule-associated protein tau (Tau-441, Sigma) was used. Tau molecules were diluted in BRB80 (final concentration of 1 μg/ml) prior to use in the experiments.

Setup and Procedure

Three different cases of MT immobilization were investigated: attachment on a glass by poly-L-lysine (PLL), attachment on a glass by inactive kinesin, and bridging between parallel walls. Experiments were performed in flow cells with different treatments of the lower cover slip. For the first case, the cover slip was coated with PLL (0.01 % PLL, 5 minutes incubation) for MT attachment (Figure 1a). For the second case, the surface was coated with inactive kinesin (50 μg/ml, 5 minutes incubation). Kinesin molecules were attached on the surface via their tail and their heads were attached to MTs for immobilization (Figure 1b). The third case required simple micro machining techniques (lithography, evaporation, etching) to fabricate parallel walls for MT-suspension in between (Figure 1c). [4] A fluoropolymer (CYTOP, Asahi Glass) was patterned into 2 μm-high and 8 μm-wide parallel walls with a distance of 22 μm between the walls. After immobilization of the MTs, kinesin-coated beads (0.49 μm) were injected in the flow cells. Adding 1mM ATP solution activated kinesin molecules. Moving beads were tracked and analyzed to measure the average transport velocities.

Performance test for molecular detection was applied to the attached (on PLL-coated surface) and the suspended (between parallel walls) cases. The average kinesin-coated bead velocities along tau-exposed MTs were measured

for each case. To perform this process, a tau protein solution (1 μg/ml in BRB80) was injected into the flow cell and incubated for 5 minutes after MT immobilization. Unbound tau was washed away with BRB80 solution. Then, the kinesin-coated beads were injected in the flow cell and their motion was monitored. In addition, motion along MTs (without tau exposure) was performed as control experiments. No-tau cases (control experiments) provided MTs without any obstacles for kinesin motion (Figure 2a). However, tau molecules hinder kinesin motion because they bind on the MT protofilaments where kinesin molecules move (Figure 2b). [5]

A photometrics camera (CascadeII 512) was used to monitor the kinesin-coated bead motion visualized under an inverted microscope (Olympus IX71) with differential interference contrast setup. Images were stored as 1-fps-movies and processed by imaging software (Metamorph, Molecular Devices, LLC).

Analysis

Moving beads (recorded in 1 fps movies) were tracked using MoveTR32 software (Library CO.). There were two criteria for the selection of the "moving beads": (1) minimum continuous motion of 15 seconds and (2) maximum pause of 2 seconds during motion. Beads satisfying these conditions were selected as "moving beads". For each "moving bead", the fastest portion of the recorded motion (≥15 seconds) was examined and the average velocity was calculated. The mean velocities for each experimental case and condition were calculated and compared. Statistical significance was calculated using student *t-test*.

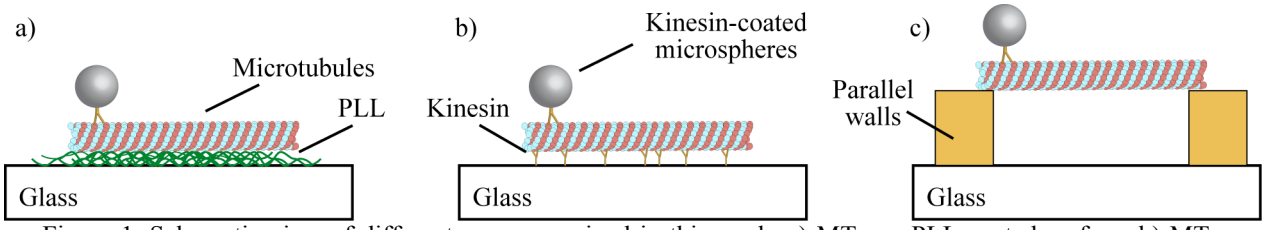

Figure 1. Schematic view of different cases examined in this work. a) MTs on PLL-coated surface, b) MTs on kinesin-coated surface and c) suspended-MTs were used as rails for kinesin-coated beads. Average velocities of kinesin-coated beads were measured after tracking the motion along MTs.

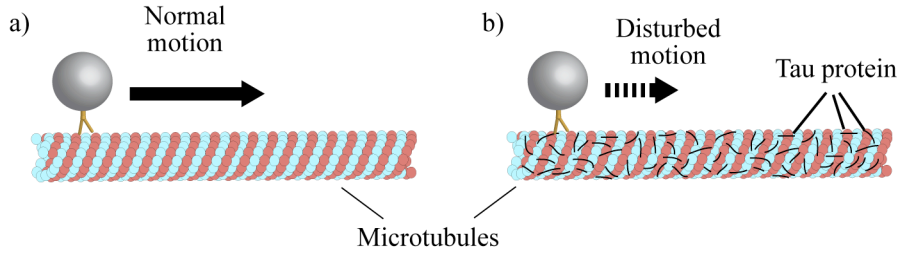

Figure 2. Schematic showing kinesin motion along a) MTs and b) tau-attached MTs. Kinesin moves freely along MTs. As tau hinders kinesin motion, average velocity along tau-attached MTs is expected to be slower.

Results and Discussion

Kinesin-coated bead motion was investigated along MTs immobilized with three different methods: attachment on a glass by PLL, attachment on a glass by inactive kinesin, and bridging between parallel walls. Average velocities of the tracked beads were measured as 0.21±0.01 μm/s (mean±SEM, n=53) along MTs attached on a PLL-coated surface, 0.28±0.01 μm/s (n=71) along MTs attached on an inactive kinesin-coated surface and 0.33±0.01 μm/s (n=51) along suspended MTs (Figure 3). The motion along MTs immobilized on a PLL-coated surface resulted in the slowest motion while the motion along suspended MTs was the fastest.

We tested the molecular detection performance along MTs attached on a PLL-coated surface (attached MTs) and MTs bridged between parallel walls (suspended MTs). For both cases, bead velocity was compared on untreated MTs (control) and on MTs that were briefly exposed (5 minutes) to 1 μg/ml solution of tau protein. The average kinesin-coated bead velocity was measured depending on the selection criteria. A decrease of average velocity between motion along tau-exposed MTs and non-tau exposed MTs was detected, indicating the tau binding to MTs.

The change in velocity was more pronounced in the suspended case although the same experimental procedure was used for both cases (Figure 4). The average velocity along tau-exposed attached and suspended MTs were 91.1% and 89.8% of the corresponding normal control, respectively (n=39 for each case). The decrease in the normalized average velocities did not show extreme difference between two cases (1.3%). However, the suspended MTs demonstrated higher significance (p=0.014) than the attached MTs (p=0.065). The significant difference with 95% confidence was achieved only along the suspended MTs. The difference between the *t-tests* indicated that suspended MT based system provided much higher sensitivity compared to the attached MT (on PLL-coated surface) case.

One possible reason why motion along suspended MTs (Figure 1c) was the fastest of all three cases could be due to existence of twisted microtubules. [6] *In vitro* polymerization of MTs frequently results in different number of protofilaments e.g. 12 or 14, causing twist in the microtubule lattice. Usually, *in vivo* assembled microtubules are

composed of 13 protofilaments, along which kinesin molecules move straight. However, twisted microtubules cause kinesin molecules to move around the axis of the MTs. For suspended MTs, 0.49 μm beads could freely move around MTs located at 2 μm above the surface. On the other hand, motion along attached MTs, might be disturbed by a glass surface, which can block the motion of kinesin molecules. When a kinesin molecule on a bead encountered glass surface, the bead could pause until another kinesin molecule on the bead took the lead and resumed the motion on a different protofilament. As a result, average velocity of the kinesin-coated bead motion along the suspended MTs was faster since kinesin switching due to twisted MTs was not an issue.

In the case of PLL-coated surface, electrostatic interaction might cause additional effect on the kinesin motion. PLL is a polycation that can bind on negatively charged MTs. Similarly, kinesin heads are positively charged and they bind on the negatively charged MTs with electrostatic interaction. [7] Therefore, when kinesin heads are in the close proximity of the PLL coating, kinesin heads might be repelled disturbing the motion. This could be one of the reasons why motion along PLL-coated surface was the slowest of all conditions.

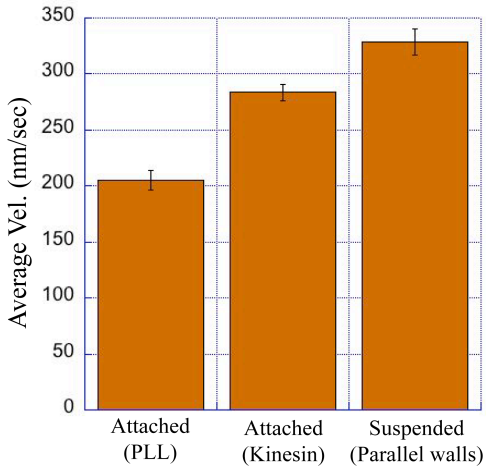

Figure 3. Graph showing the average transport velocities of kinesin-coated beads for different immobilization conditions. Motion along microtubules on PLL coating, kinesin coating and suspended microtubules were performed. n = 52, 71 and 57 respectively.

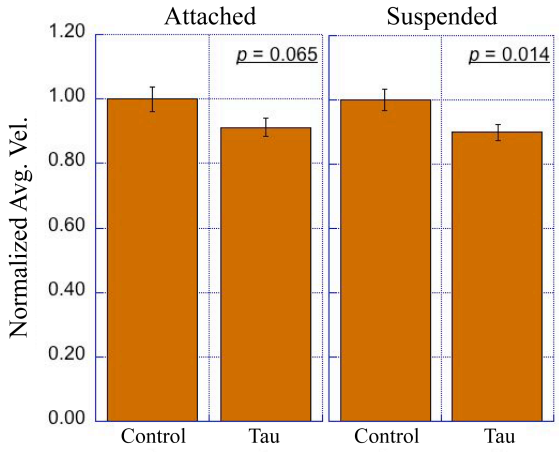

Figure 4. Graph showing average transport velocities along tau-exposed MTs and non-tau exposed MTs. Two different cases (attached MTs and suspended MTs) were examined. As the average velocities of the attached case were slower, for better comparison, normalized velocities were used. *p* values of t-tests show higher significance of the suspended MT case (n = 39 for each case)

Conclusion

We have proposed a new geometry for MT-based bead assay, i.e. suspended MTs, and investigated three different MT immobilization: (1) on a PLL-coated glass surface, (2) on an inactive kinesin-coated glass surface, and (3) between micromachined parallel walls. Kinesin-coated bead motion along suspended MTs was the fastest while kinesin-coated bead motion along attached MTs on a PLL-coated surface was the slowest. Then, we have compared those two cases based on their performance on protein detection. Proposed suspended MTs provided higher sensitivity. As MTs are essential components of intraneuronal transport, higher sensitivity in a reliable *in vitro* system utilizing MTs leads to detection systems used for drug discovery and diagnostic devices of diseases such as neurodegenerative diseases.

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