Supporting Information

Direct Observation of Growth and Shrinkage of Microtubules by Single Molecule Förster Resonance Energy Transfer

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1. Experimental Section:

1.1 Materials: Diamino-polyethylene glycol with MW 2000 Da (PEG2000) was purchased from Rapp Polymer. (3-Glycidoxypropyl)trimethoxysilane (GOPTS) was purchased from Fluka. EZ-Link NHS-Biotin was purchased from Thermo Scientific. Hydrogen peroxide (30% solution), Acetone, Dichloromethane and N, N-Dimethylformamide were purchased from Merck. Imidazole and 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid (HEPES) were purchased from Himedia. 1,4-Piperazinediethanesulfonic acid (PIPES), ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), guanosine-5’-triphosphate sodium salt hydrate (GTP), β-casein, catalase, glucose oxidase and β-mercaptoethanol were purchased from Sigma Aldrich. Alexa Fluor 568 carboxylic acid succinimidyl ester was purchased from Invitrogen. Guanosine-5’-[(α,β)-methyleno]triphosphate Sodium salt (GMPCPP) was purchased from Zena Bioscience.

1.2 Protein Biochemistry: Tubulin was isolated from goat brain and it was labelled with biotin and alexa Fluor 568 using EZ-Link NHS-Biotin and alexa fluor 568 carboxylic acid succinimidyl ester respectively to obtain the biotin and alexa-568-labelled tubulin. Purification of tubulin from goat brain, alexa-568 labelling of tubulin, and the polymerization of microtubules were carried out following literature procedure. For tubulin labelled with alexa 568, dye per protein ratio was determined spectrophotometrically to be 1.1. This ensures the presence of single alexa 568 molecule in a single tubulin. Unlabelled tubulin was stored in liquid nitrogen at a concentration of 200 μM and labelled tubulin at concentrations of 150 μM. Tubulin concentrations represent tubulin dimer concentrations. Plasmids of deca-histidine tagged EGFP (EGFP-His10) and Mal3-EGFP proteins (received as gift from Dr. Thomas
Surrey, Cancer Research UK) were expressed in E-coli and purified through Ni-NTA column in our laboratory following previously described method.\textsuperscript{2} For Mal3-EGFP, the expression vectors were created by inserting a sequence coding for a linker (ILGAPSGGGATAGAGGAGGPAGLIN) and then the sequence for GFP was inserted. This results into a C-terminally tagged constructs of Mal3-EGFP.\textsuperscript{2b}

1.3 Preparation of biotin functionalized Surface:\textsuperscript{2a,3} Glass coverslips (50X50 mm) were cleaned with 3M NaOH, followed by sonication for 30 minutes. Next, the coverslips were cleaned with plenty of water and further treated with piranha (2:3 mixture of hydrogen peroxide and sulphuric acid) followed by ultrasonication for 45 min under fume hood. Piranha solution was discarded and glass slides were thoroughly cleaned with water and dried under stream of nitrogen gas. Surface functionalisation was achieved by following stepwise method.\textsuperscript{3} Silanisation of glass surfaces has been achieved by treating with GOPTS at 75 °C. Next, silanised glass surfaces were treated with diamino-polyethylene glycol and heated at 75 °C for overnight. The polyethylene glycol functionalised surfaces were washed with plenty of water for complete removal of excess and unreacted diamino-polyethylene glycol from surfaces. Finally, polyethylene glycol surface was treated with Biotin-NHS for 1 hour at 75 °C. Biotin functionalised surfaces were washed with DMF and plenty of water followed by drying under stream of air.

1.4 Biotin labelled and unlabelled Alexa 568-GMP-CPP Microtubule preparation:\textsuperscript{3}

Preparation of biotin and alexa fluor 568 labelled GMP-CPP microtubules:

(A) *Tubulin mix on ice:* 0.75 μL Alexa Fluor 568 labelled tubulin (15 mg/mL, 65% labelling ratio), 0.75 μL biotin labelled tubulin, 4 μL tubulin (20 mg/mL), 44.5 μL BRB80 were mixed on ice.

(B) *Final mix on ice:* 5 μL GMP-CPP (10 mM), 1 μL MgCl\textsubscript{2} (100 mM), 10 μL tubulin mix, 34 μL BRB80 were mixed and incubated for 2 hour at 37 °C.

(C) The final mix was centrifuged for 7 min at 14,000 rpm in a table top centrifuge. The colored pellet was resuspended in 50 μL warm BRB80 (37 °C).

Alexa fluor 568 labelled GMP-CPP microtubules were prepared as described above. The only change in step 1A was 1.5 μL alexa fluor 568 labelled tubulin added instead of biotin labelled tubulin.

1.5 Immobilisation of GMP-CPP microtubules labelled with biotin and Alexa-568 and FRET study in presence of Mal3-EGFP:

A brief overview of the experimental condition is as follows. First, a flow chamber of around 5 μL was constructed from a biotin-PEG functionalized glass and one poly-L-lysine (PLL)-PEG passivated counter
glass, separated by two strips of double sticky tape (Tesa, Hamburg, Germany). The flow chamber was equilibrated with 20 μL of BRB80 (80 mM PIPES, 1 mM EGTA, 1 mM MgCl$_2$ pH 6.8) while positioned on an ice-cold metal block, then with 20 μL of BRB80 containing 1 mg mL$^{-1}$ β-casein for 5 min. Unbound β-casein was washed out by BRB80. Next, neutravidin 50 μg mL$^{-1}$ was flowed into the flow chamber, followed by incubation for 5 min. Unbound neutravidin was washed out by BRB80 and flow-chamber was allowed to warm up to room temperature (30 °C). Then the flow chamber was incubated with pre-formed GMP-CPP stabilized alexa-568 and biotin labelled microtubules in BRB80 containing 3 mM Mg-GTP (13% labelling ratio of alexa 568 per tubulin) for 5 min. Unbound microtubule seeds were washed out using previously described warm buffer and immediately flow chamber was filled with 23.07 μM alexa 568 tubulin mix (13% labelling ratio of alexa-568 per tubulin) and 85 nM Mal3-EGFP in final buffer (BRB80 supplemented with 2 mM GTP, 7 mM MgCl$_2$, 10 mM 2-mercaptoethanol, 0.15% methylcellulose and an oxygen scavenger system (50 mM glucose, 1 mg mL$^{-1}$ glucose oxidase, and 0.5 mg mL$^{-1}$ catalase)) on room temperature and transferred to the microscope followed by acquisition of data at 37 °C (maintained through a Tokaihit stage top incubator). Control experiment using EGFP-His$_{10}$ instead of Mal3-EGFP was performed following above described experimental method. Each experiment was carried out on 20 different microtubules.

2. Instrumental Set-up and Data Analysis

2.1 Emission Spectra under a Confocal Microscope

An electron multiplying charge-coupled device (EMCCD, ANDOR Technology) and a spectrograph (ANDOR Technology, Shamrock series) have been used to record the steady state emission under the microscope. The spectrograph is attached to one of the ports of the PicoQuant MicroTime 200 apparatus.

2.2. Confocal Microscopy: FRET

The confocal setup (PicoQuant, MicroTime 200) with an inverted optical microscope (Olympus IX-71) is described earlier. The donor (EGFP) was excited at 470 nm using a picosecond diode with stable repetition rate (40 MHz). The power of the exciting laser was kept at 30 μW during FRET measurements. The fluorescence was separated from the exciting laser with a dichroic mirror (490DCXR, Chroma) and appropriate band-pass filters (HQ500lp, Chroma).

The donor and the acceptor fluorescence signal were captured separately using a dichroic mirror (540DCLP) and two detectors (Micro photon device, MPD). Two additional band pass filters (FF01-520/35 for the donor and 600 nm band pass for the acceptor) were used to further separate the donor and acceptor fluorescence.
R₀ (Förster distance) can be determined from the spectral overlap, J(\lambda) between the donor emission and the acceptor absorption as follows.⁵

\[
J(\lambda) = \frac{\int_{0}^{\infty} F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda}{\int_{0}^{\infty} F_D(\lambda) d\lambda}
\]

(1)

\[
R_0 = 0.211 \left[ \kappa^2 n^{-4} Q_D(\lambda) \right]^{1/6}
\]

(2)

In this work, we used the reported R₀=49 Å for the EGFP-alexa 568 system.⁶

3. Fluorescence Correlation Spectroscopy (FCS) Data

The autocorrelation function of EGFP labelled Mal3 is fitted according to the following equation.

\[
G(\tau) = \left[ \frac{1}{N} \right] \left[ 1 + \frac{\tau}{\tau_D} \right]^{-1} \left[ 1 + \frac{\tau}{\omega^2 \tau_D} \right]^{1/2} \left[ 1 - \sum_i A_i - A_i \exp \left( -\frac{\tau}{\tau_{Ai}} \right) \right] \left[ 1 - \sum_i A_i \right]
\]

(3)

where N is the number of molecules in the observed volume, \( \tau_D \) is the diffusion time of species, \( \omega \) is the height-to-diameter ratio of the 3D Gaussian confocal volume and \( \tau_{Ai} \) is the relaxation time for an exponential component with an associated amplitude \( A_i \). For data analysis we used the IgorPro 6 software.

FCS data for Mal3-EGFP displays a diffusion time (\( \tau_D \)) of ~ 550 µs. Diffusion coefficient (\( D_t \)) of Mal3-EGFP is calculated using the following equation.

\[
D_t = \frac{\omega^2}{4 \tau_D}
\]

(4)

From this, \( D_t \) of Mal3-EGFP is found to be ~ 46 µm² s⁻¹. Assuming Mal3-EGFP to be a spherical molecule, the hydrodynamic radius (\( r_H \)) of Mal3-EGFP may be calculated by applying Stokes-Einstein equation as follows.

\[
D_t = \frac{kT}{6\pi \eta r_H}
\]

(5)

Where, \( r_H \) is the hydrodynamic radius of a molecule diffusing in a medium of viscosity \( \eta \). Using the value of \( D_t \) (Figure S1) of Mal3-EGFP \( r_H \) is calculated to be 45±2 Å. Thus diameter of Mal3-EGFP is ~90 Å.
**Figure S1.** Kymographs from one movie indicates Mal3-EGFP binds on dynamic microtubules grows from GMP-CPP seeds. Scale bar corresponds to 2 µm.

**Figure S2.** Normalized FCS trace of Mal3-EGFP. Red hollow circles indicate data points and solid black line indicates best fit.
References


