Resolving bundled microtubules using anti-tubulin nanobodies

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Microtubules are hollow biopolymers of 25-nm diameter and are key constituents of the cytoskeleton. In neurons, microtubules are organized differently between axons and dendrites, but their precise organization in different compartments is not completely understood. Super-resolution microscopy techniques can detect specific structures at an increased resolution, but the narrow spacing between neuronal microtubules poses challenges because most existing labelling strategies increase the effective microtubule diameter by 20–40 nm and will thereby blend neighbouring microtubules into one structure. Here we develop single-chain antibody fragments (nanobodies) against tubulin to achieve super-resolution imaging of microtubules with a decreased apparent diameter. To test the resolving power of these novel probes, we generate microtubule bundles with a known spacing of 50–70 nm and successfully resolve individual microtubules. Individual bundled microtubules can also be resolved in different mammalian cells, including hippocampal neurons, allowing novel insights into fundamental mechanisms of microtubule organization in cell- and neurobiology.
Microtubules are hollow biopolymers of 25-nm diameter and are key constituents of the cellular cytoskeleton, the mechanical framework of dynamic polymers and associated proteins that directs cell shape and facilitates intracellular transport. The exact spatial organization of microtubules and their bundling is of central importance to a number of fundamental cellular processes such as mitosis, cell polarization and the outgrowth of cellular processes, for example, in neurons. Conventional fluorescence microscopy allows selective labelling of microtubule modifications and associated proteins, but cannot resolve individual microtubules within tightly bundled microtubule arrays. Electron microscopy, in contrast, allows resolving individual microtubules, but is very labour intensive, while high-density labelling of specific proteins has remained challenging. Single-molecule localization microscopy (SMLM) provides selectivity at an increased resolution, but the extremely small spacing between neuronal microtubules (20–70 nm) poses novel challenges, because existing labelling strategies typically increase the apparent microtubule diameter by 20–40 nm and will thereby blend neighbouring microtubules into one structure. It is therefore widely assumed that despite all progress in super-resolution microscopy, electron microscopy is still the only technique that allows insight into complex microtubule structures. Here, we use both computer simulations and experimental approaches to explore how labelling strategy affects SMLM imaging of microtubules. We develop single-chain antibody fragments (nanobodies) against tubulin and achieve super-resolution imaging of microtubules with a decreased apparent diameter, allowing us to optically resolve bundled microtubules.

Results
Simulations of microtubules with different labels. To explore the effect of label size and fluorescent probe positioning on resolving ability, we first performed numerical simulations to examine how labelling density, localization precision and fluorophore positioning affect the apparent microtubule width (determined as the full width at half maximum (FWHM) from Gaussian fits to intensity profiles integrated over 512 nm of microtubule length; Fig. 1a). Using a maximum localization uncertainty of 8 nm, we found that the apparent microtubule width was ~31 nm for a fluorophore positioned directly at the microtubule surface (probe position of 0 nm, Fig. 1b). Placing the fluorophore further away increased the FWHM by double the displacement, that is, 41 nm for a fluorophore position of 5 nm. A more stringent precision cutoff resulted in decreased FWHM (Fig. 1c) and the FWHM decreased from 63 nm for a probe position of 15 nm and precision cutoff at 13 nm to 27 nm with fluorescent probes directly on the microtubule lattice and a precision cutoff of 3 nm.

To examine how label size affects the probability of resolving closely spaced microtubules, pairs of randomly picked profiles were superimposed with a set distance between the microtubule centres and the resulting profile was analysed. If the lowest intensity between the two microtubule centres was <75% of the intensity of the lowest peak, then the microtubules were considered to be resolved and the resolving probability was calculated as the fraction of resolvable cases out of 250. As expected, decreasing label size results in increasing the resolving probability (Fig. 1d). For example, given a labelling density of 7% and a precision cutoff of 13 nm, the probability of resolving microtubules with centres spaced 55-nm apart increased from 0.03 to 0.49 to 0.97 for probes positioned at 12.5 nm, 5 nm and 0 nm from the microtubule lattice, respectively (data taken from fit).

Generation and characterization of tubulin nanobodies. Conventional staining strategies often use a combination of primary antibodies binding a specific epitope, followed by a fluorescently tagged secondary antibody that recognizes the primary antibody, resulting in significant displacement of the fluorescent probe from the target (Fig. 1e). Typically, smaller labels have been obtained by directly conjugating a fluorophore to the primary antibody, or by using antibody fragments. Antibody fragments derived from heavy chain only camelid antibodies (nanobodies) are now emerging as promising alternatives, because of their small size (~15 kDa, ~4 nm), as well as ease of selection and production. Previous work has demonstrated the usage of nanobodies to create smaller labels for SMLM. Overexpression of GFP–tubulin and subsequent labelling with an anti-GFP nanobody conjugated to a fluorescent dye significantly decreased the effective diameter of individual microtubules. However, this strategy requires overexpression of GFP–tubulin to very high levels, which will perturb cytoskeletal organization and is not possible in many biological systems.

To experimentally assess the effect of label size on resolving power, we created three novel labels for SMLM of endogenous tubulin, complementing the existing strategies using conventional antibodies. First, we developed two different nanobodies against tubulin. One was derived from two rounds of phage display selection using a universal synthetic library of humanized nanobodies (VHH#1) and the other using an MRC7 cell library (VHH#2) (Supplementary Fig. 1a,b; see Methods section for details), similarly selected in two rounds of phage display. Immunoblotsing with VHH#1 or VHH#2 on lysates of HEK293 cells overexpressing GFP–α-tubulin or GFP–β-tubulin revealed that both nanobodies react with the endogenous tubulin as well as GFP–β-tubulin (Supplementary Fig. 2a,b). Conjugation of Alexa Fluor 647 (AF647) to the nanobodies did not interfere with their binding properties (Supplementary Fig. 2c; Supplementary Figs 3 and 4). As a second approach, recombinant human-derived single-chain variable fragments (scFvs) directed against α- and β-tubulin were purified and also coupled to AF647 (ref. 5). All bacterially expressed and purified labels were relatively pure and stable over long periods of time (Supplementary Fig. 2a).

Resolving microtubule bundles in vitro. To test the SMLM resolving power of the different microtubule labels, we established an in vitro bundling assay using polymerized microtubules in combination with the microtubule bundler AtMAP65-1, which promotes the formation of a planar network of antiparallel microtubules with a single-dimer spacing in between (Fig. 1f). Silanized coverslips were used to stably attach the microtubule bundles to the coverslip surface to allow for subsequent staining procedures. As a control, we performed SMLM on non-stained samples to which fluorescently tagged tubulin (conjugated to HiLyte Fluor 647) was added in to the polymerization mix. In this condition, most bundles could be clearly resolved with an average spacing of 65 ± 2 nm (s.e.m., n = 56, Supplementary Fig. 3a). Both VHH#1 and VHH#2 conjugated to AF647 efficiently decorated the bundles and in most cases the individual microtubules could be clearly distinguished when the microtubule centres were 60–70-nm apart (Fig. 1g, Supplementary Fig. 3b). In contrast, when a conventional primary anti-α-tubulin antibody directly coupled to AF647 was used, such bundled microtubules could often not be resolved.

Comparative analysis of microtubule labels in adherent cells. When we tested our nanobodies on microtubules in cells, we found that we could resolve microtubules that were spaced down to 40 nm (Fig. 2a). To quantitatively compare the nanobody...
Figure 1 | Smaller labels allow resolving bundled microtubules. (a) Simulations of conventional (top) and single-molecule localization-based microtubule images for different probe densities, localization precision cutoffs and probe positions (distance between target molecule and fluorophore). Unless specified otherwise, probe position is 2.5 nm and precision cutoff is 8 nm. Probe density is 100% and 50% for the third and fourth row, respectively. A Gaussian localization accuracy distribution with mean ± s.d. of 7.5 ± 2.5 nm is used. (b) FWHM of Gaussian fits to microtubule cross sections integrated over 512 nm length as a function of probe density and for different probe positions. Error bars represent s.e.m. Each point is the average of 150 FWHMs measured on 512 nm long microtubule (MT; empty stretches along the MT were not included). (c) MT FWHM versus probe position for different cutoffs of the localization accuracy distribution. (d) Estimation of resolving power for staining of microtubules with probes at increasing distance from the microtubule. Probe density is 7%, localization precision cutoff threshold is 13 nm. Two-hundred and fifty profiles per distance. (e) Illustration of the different labelling strategies compared in this study. (f) Scheme of the in vitro microtubule bundling assay to test the resolving power of different microtubule labelling strategies. Rhodamine-labelled microtubules are assembled into planar bundles with defined spacing formed by the microtubule-bundler GFP-AtMAP65-1. (g) Conventional (top) and SMLM (middle and bottom left) images and representative line scans (bottom right) of in vitro microtubule bundles stained with a fluorescently labelled primary anti-α-tubulin antibody (1ary-AF647) or two novel tubulin nanobodies (VHH#1 and VHH#2) conjugated to AF647. Scale bar, 1 μm. More examples are provided in Supplementary Fig. 3.

approach with the other staining methods in cells, we labelled microtubules in fixed Ptk2 and COS-7 cells using different tubulin labels conjugated to AF647. We determined the FWHM from a Gaussian fit to intensity profiles perpendicular to the microtubule averaged over 512-nm length to rule out possible profile artifacts that could arise from low labelling density (Fig. 1b). We found that individual microtubules were densely labelled with the most common diameter (average mode ± s.e.m.) varying from 39.3 ± 0.8 nm (VHH#2, N = 10 data sets with in total n = 1,365 profiles) to 54.0 ± 1.2 and 61.7 ± 0.8 nm (directly conjugated primary anti-tubulin antibody, N = 10, n = 2,462, and primary anti-tubulin + secondary-AF647, N = 10, n = 2460, respectively; Fig. 2b, Supplementary Fig. 4a,b; see Methods section for details and Supplementary Fig. 5a for statistical testing). Because in the rendering of the SMLM images we rejected all localizations with localization precision ≥ 13 nm, these values suggest that fluorophores coupled to primary antibodies are on average ~ 12.5-nm displaced from the microtubule lattice (Fig. 1c). Strikingly, this distance is reduced to < 2.5 nm for VHH#2.

To translate the observed microtubule FWHM into a resolution estimate, we again analysed composite profiles obtained by superimposing two randomly picked profiles with a set distance between the microtubule centres (Fig. 2c). On the basis of cumulative probability plots obtained for the VHH#1, VHH#2, primary and primary–secondary antibody labellings, ~ 50% of all bundled microtubules with 25-nm lattice-to-lattice spacing (corresponding to 50 nm between peaks) will be resolved
Figure 2 | Resolving bundled microtubules in cells using tubulin nanobodies. (a) SMLM reconstruction of a Ptk2 cell stained with VHH#1 and intensity profile of closely spaced microtubules along the yellow line. Yellow arrows indicate microtubule ends. Scale bar, 1μm. A larger field of view of the same cell can be found in Supplementary Fig. 4b. (b) Histograms of microtubule FWHM for different probes. scFvs: mixture of human single-chain antibody fragments (scFvs) recognizing α- and β-tubulin. For representative images, see Supplementary Fig. 4a. (From top to bottom: n = 1,365, 547, 352, 2,462, 2,460 profiles from N = 10, 5, 9, 10, 10 different acquisitions). Mean (blue) and mode (red) value are indicated ± s.e.m. (using N). (c) Estimation of resolving power for different labels obtained by combining arbitrarily selected line profiles at increasing distance between centres. (d) Scatter plot of FRC resolution estimate versus microtubule FWHM for images of microtubules in COS-7 cells stained with different labels. Error bars depict 95% confidence intervals. (e) Overview 3D-SMLM reconstruction of a U2OS cell stained with AF647-labelled VHH#1. The z-depth is colour-coded according to the scale on the left of the image. Scale bar, 5μm. (f) Magnified image of the inset in (e). Colour code is the same as in (e). Scale bar, 500 nm. (g) Area containing parallel microtubules at different depth in the cell. Colour code is the same as in (e). Scale bar, 500 nm. (h) Collapsed cross section (z-x) of the volume depicted in (g). Scale bar, 100 nm. (i-k) SMLM reconstruction of microtubule bundles labelled with VHH#1 in the dendrites of a hippocampal primary neuron. Yellow arrows indicate microtubule ends and yellow lines were used for line scans across densely packed microtubule bundles (j,k). Inset shows the diffraction-limited fluorescence image. Scale bar, 2μm. (l) 3D-SMLM reconstruction of a hippocampal primary neuron labelled with VHH#1. The Z-depth is colour-coded according to the scale on the left of the image. Yellow arrows indicate microtubule ends. Inset shows the diffraction-limited fluorescence image. Scale bar, 2μm.
by the nanobody labels, whereas the directly conjugated primary antibodies or the sandwich labelling will only resolve ~20% and ~5% of all microtubule pairs, respectively. Consistent with the in vitro bundling results, VHHs are expected to resolve >90% of microtubule pairs with a lattice-to-lattice spacing of 60 nm, which is the typical spacing of tightly bundled microtubules in neuronal dendrites2. To further quantify the gain in resolution, we used the Fourier Ring Correlation resolution measure (FRCrm) as an independent, quantitative estimate of resolution that accounts for both localization precision and probe density7. Whereas direct application of the available FRC ImageJ-plugin to our data yielded highly variable results, this could be circumvented by data preprocessing to average different localizations emerging from the same fluorophore emitting over multiple frames (Supplementary Fig. 5b). As expected, smaller apparent diameters also resulted in better FRCrm resolution estimates, with the exception of VHH#2, whose lower labelling density resulted in a worse FRCrm compared to VHH#1, despite its smaller FWHM. For VHH#1, the average FRCrm was 45 ± 4 nm (Fig. 2d). These results demonstrate that our novel anti-tubulin nanobodies provide improved resolution.

**Tubulin nanobodies for 3D-SMLM in U2OS cells and neurons.** To test how anti-tubulin nanobodies performed in three dimensional (3D)-SMLM, we labelled microtubules in U2OS cells with VHH#1 and performed 3D-SMLM using the biplane approach8. We found that microtubules could easily be resolved in z-direction (Fig. 2e–h) at distances of 100 nm. Finally, we used the VHH#1 nanobody to perform SMLM on microtubules in primary hippocampal neurons (Days in vitro, DIV1) and could successfully resolve individual microtubules in neurites (Fig. 2i–l). The cross-sections across densely packed microtubule bundles indicate a center-to-center spacing of 60 to 80 nm, consistent with earlier results using electron microscopy on cross sections9. In several cases, ends of individual microtubules could be clearly identified (Figs 2i,l, arrows). Thus, tubulin nanobodies can be used to resolve neuronal microtubule bundles.

**Discussion**

We have introduced novel labels for microtubules that allow using SMLM to resolve previously inaccessible functional details of microtubule organization such as bundling, both in vitro and in fixed cells. These labels nicely complement the recently introduced live-cell marker for tubulin8 that allows nanoscopy in fixed cells. These labels nicely complement the recently introduced live-cell marker for tubulin8 that allows nanoscopy in fixed cells. These labels nicely complement the recently introduced live-cell marker for tubulin8 that allows nanoscopy in fixed cells.

**Methods**

**VHH#1 selection.** VHH#1 was selected from a novel library of 3 × 109 humanized nanobodies. Briefly, commercial biotinylated tubulin (Cytoskeleton) was diluted to obtain a ~20–200 nm solution (1 ml final) and efficient recovery of biotinylated tubulin was confirmed on 50 μl streptavidin-coated magnetic beads (Dynal). Fractions of bound and unbound samples were compared by western blot using streptavidin-HRP. Adequate amounts of beads and biotinylated antigen were incubated for 2 h with the phage library (1010 phages diluted in 1 ml of PBS containing 0.1% Tween-20 and 2% nonfat milk). Phages were previously adsorbed on empty streptavidin-coated magnetic beads to remove nonspecific binders.

To obtain bound to tubulin-coated beads, microtubules were recovered on a round-bottomed microfuge tube, washed 10 times (round 1) or 20 times (round 2) using PBS containing Tween-20 0.1%. Bound phages were eluted using 500 μl triethylamine (100 mM) for 10 min. Eluted phages were neutralized using 1 M Tris pH 7.4. Elution was repeated once more. E. coli (TG1) were infected with the eluted phages. Round 2 was carried out using 1012 phages as input. After round 2, 40 bacteria clones were picked at random and used to produce nanobodies in the culture medium. Nanobody specificity was analysed by immunofluorescence as described before and nanobodies staining microtubules were analysed further.

**VHH#1 expression and purification.** For production of VHH#1, WK6 E. coli containing the plasmid pHEN2−VHH#1−His6−cMyc2 were grown in 21 of Terrific Broth (17 mM KH2PO4, 72 mM K2HPO4, 12 g l−1 tryptone, 24 g l−1 yeast extract, 0.4% glycerol) containing 2 mM MgCl2, 0.1% glucose, and 100 μg ml−1 ampicillin with shaking at 37 °C until the E. coli had an OD600 of 0.6–0.9. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a concentration of 0.5 mM, and the flasks were shaken at 28 °C overnight (~16 h). To extract the nanobody from the periplasmic space, cells were centrifuged (5,000 g, 10 min), resuspended in 24 ml of TES buffer (0.2 M Tris pH 8.0, 0.5 mM EDTA, 0.5 μM sucrose) and shaken for 1 h at 4 °C. The cell–TES mixture was then diluted by the addition of 36 ml of TES/4 buffer (50 mM Tris pH 8.0, 0.125 mM EDTA, 0.125 M sucrose), and shaken for 1 h at 4 °C. The cells were then pelleted (5,000 g, 10 min), and the nanobody-containing supernatant removed. The His6-tagged VHH#1 was then purified using HisPur cobalt-agarose resin (Thermo Scientific) following manufacturer’s instructions. The eluted protein was concentrated ~10-fold using ‘Vivaspin’ columns (3 kDa MWCO; General Electric). SDS–polyacrylamide gel electrophoresis (PAGE) and Coomassie-staining of the resulting gels revealed the nanobody to be >90% pure. VHH#1 was dialysed overnight against PBS at 4 °C to remove any residual imidazole. The 21 of culture yielded ~50 mg of pure nanobody. The stability of VHHs was analysed by SDS–PAGE. After SDS–PAGE, the protein was subjected to the second round of selection with 0, 0.1, 1 or 5 μg of tubulin. E. coli TG1 were infected with the phages from the second selection and plated on LB agar. Phages were recovered by plating on iron-deficient LB agar plates supplemented with ampicillin. Ninety-six random colonies were picked for testing. Expression of VHHs targeted to the bacterial periplasm was induced by addition of 1 mM IPTG at 37 °C overnight. To obtain the periplasmic fraction, bacterial pellets were resuspended in 10 volumes of PBS (pH 7.4) containing protease inhibitor cocktail (Roche), subjected to two freeze/thaw cycles, and spun down for 15 min at 4,600 g. Periplasm was collected as supernatant fraction. Specificity of VHHs for tubulin was determined by enzyme-linked immunosorbent assay.

**VHH#2 expression and purification.** For efficient bacterial expression, four of the most successful and divergent VHH sequences were directly cloned from pUR8100 into modified pET28a−EPEA vector using SfiI/NotII restriction sites. pET28a−EPEA was created inserting AACAAGAQGYDYEPEA−STOP sequence (NotII/NotI) in front of the C-terminal 6 × His-myc sequence which allows purification with Capture Select C-tag matrix (Life Technologies). Although all of the constructs were expressed and purified, from now on, we focused on one of the VHH sequences showing the best performance during protein production and labelling (Clone H, that is, VHH#2).

For protein production, an overnight culture of E. coli BL21(DE3) transformed with pET28a−VHH#2−EPEA was grown in LB supplemented with kanamycin till OD600 ~0.8 and induced with 0.5 mM IPTG for 4 h at 25 °C or at 20 °C overnight. VHHs were purified from the periplasmic fraction in PBS (pH 7.4) containing 0.5% Triton-X100, protease inhibitor cocktail (Roche) and 0.5 mM TCEP and purified using Capture Select C-tag matrix according to the manufacturer’s instructions (Life Technologies). Bound VHH was eluted from the beads in buffer containing 20 μl per 0.1 M Tris-HCl pH 7.4 and imidazole and PBS (pH 7.4). Impurities were removed by size exclusion chromatography performed on an AKTA FPLC system (AKTA purifier, GE Healthcare, UK) using a Superdex 200 26/60 column (GE Healthcare, UK) at a flow rate of 0.5 ml per min. Fractions of bound and unbound samples were compared by western blot using streptavidin-HRP. Adequate amounts of beads and biotinylated antigen were incubated for 2 h with the phage library (1010 phages diluted in 1 ml of PBS containing 0.1% Tween-20 and 2% nonfat milk). Phages were previously adsorbed on empty streptavidin-coated magnetic beads to remove nonspecific binders. Bound phages were eluted using 500 μl triethylamine (100 mM) for 10 min. Eluted phages were neutralized using 1 M Tris pH 7.4. Elution was repeated once more. E. coli (TG1) were infected with the eluted phages. Round 2 was carried out using 1012 phages as input. After round 2, 40 bacteria clones were picked at random and used to produce nanobodies in the culture medium. Nanobody specificity was analysed by immunofluorescence as described before and nanobodies staining microtubules were analysed further.
Cell culture and immunostaining. COS-7, MRC5 or Pt2k cells were plated on 19-mm diameter glass coverslips or 8-well Labtek chambers (Thermo scientific), respectively and cultured in DMEM/Ham’s F10 (50/50) medium supplemented with 10% FCS and 1% penicillin/streptomycin for 2-3 days. Culturing of primary neurons was described before10. Briefly, hippocampal primary neurons were prepared from embryonic day 18 rat brains. Cells were plated on coverslips coated with poly-L-lysine (10 μg/ml) and laminin (0.25 μg/ml) at a density of 40,000 per well. Hippocampal cultures were grown in Neurobasal medium (NB) supplemented with B27, 0.5 μM glutamine, 12.5 μM glutamate and penicillin/ streptomycin. For optimal microtubule imaging, cells were pre-extracted and fixed in 4% PFA/C176/glutaraldehyde for 10 min. They were washed three times with PBS, incubated with blocking buffer and added for 1-1.5 h at room temperature. Antibodies were diluted to about 1:100), AF647 conjugated goat anti-mouse IgG (H+L) secondary antibody (Molecular Probes, Life Technologies, dilution 1:300), VHH1#1 and VHH2#2 were diluted to about 10 μg/ml. All coverslips were extensively washed with PBS shortly before imaging. Live cells were post-fixed in 0.1% GA for 10 min, at room temperature and again extensively washed with PBS. For co-staining with β-actin marker, neurons already labelled with VHH1#1 and VHH2#2 were washed in PBS and incubated with AF568 Phalloidin from Molecular probes (Life Technologies, 1:2000 in PBS) for 20 min, extensively washed in PBS and mounted for imaging. For live staining, neurons were cultured for 100 min of the post-fixed condition added to the growth medium and incubated for 1h at 37°C, 5% CO2. MRC5 cells expressing plus-end microtubule marker EB3-GFP were used for the life imaging. COS7 cells were fixed with standard pre-extraction/fixation protocol (see above), mixture of 3% PFA and 1% glutaraldehyde for 10 min at 37°C or 4% PFA for 10 min at 37°C. Fixed cells were extensively washed in PBS and processed for imaging.

Pt2k cells were fixed at 37°C using prewarmed PEM buffer (15 mM PIPES pH 7.1 mM MgCl2, 10 mM EGTA) containing 0.1% Triton X-100 and 1% glutaraldehyde for 10 min. They were washed three times with PBS, incubated with PBS containing 50 mM NH4Cl for 10 min, washed twice with PBS, incubated with freshly prepared 1% GA and 1% catalase in PBS for 30 min, washed again and incubated overnight at 4°C (antibody) or 1-2 days at RT (VHHs). For the secondary antibody labelling, coverslips were washed from the primary antibody and anti-mouse antibody conjugated to AF647 were diluted in a same blocking buffer and added for 1-1.5 h at room temperature. Antibodies were diluted 1:30 in PEM80 (80 mM PIPES, pH 6.9, 2 mM MgCl2, 1 mM EGTA) containing 0.1% Triton X-100 and 0.4% glucose oxidase (Sigma-Aldrich, clone B-5-1-2, T3168) conjugated to AF647 (dilution 1:100), AF647 conjugated goat anti-mouse IgG (H+L) secondary antibody (Molecular Probes, Life Technologies, dilution 1:300), VHH1#1 and VHH2#2 were diluted to about 10 μg/ml. All coverslips were extensively washed with PBS shortly before imaging. Live cells were post-fixed in 0.1% GA for 10 min, at room temperature and again extensively washed with PBS. For co-staining with β-actin marker, neurons already labelled with VHH1#1 and VHH2#2 were washed in PBS and incubated with AF568 Phalloidin from Molecular probes (Life Technologies, 1:2000 in PBS) for 20 min, extensively washed in PBS and mounted for imaging. For live staining, neurons were cultured for 100 min of the post-fixed condition added to the growth medium and incubated for 1h at 37°C, 5% CO2. MRC5 cells expressing plus-end microtubule marker EB3-GFP were used for the life imaging. COS7 cells were fixed with standard pre-extraction/fixation protocol (see above), mixture of 3% PFA and 1% glutaraldehyde for 10 min at 37°C or 4% PFA for 10 min at 37°C. Fixed cells were extensively washed in PBS and processed for imaging.
An independent estimate of image resolution was obtained using Fourier Ring Correlation (FRC), as described previously. In short, particle tables generated by Detection of Molecules were converted to tables with only x- and y-coordinates for each localization remaining. The FRC plug-in for ImageJ created by the Delft University of Technology Quantitative Imaging Group was then used to obtain a resolution estimate. To obtain consistent results, it was essential to perform frame-to-frame fluorescent linking (see above, Supplementary Fig. 3).

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Author contributions
H.E. and L.C.K. conceived the project (independently). M.Ml. developed VHH#2, together with M.M.K., M.S. and P.M.P.v.B.e.H., who provided the MRC7 library. K.F., H.E., F.P. and S.M. developed VHH#1. M.Ma. and F.O. produced scFvs. M.Mi., B.C., R.v.d.B., and K.F. performed experiments and analysed the data. J.T. and E.K. developed simulations. M.V. contributed AtMAP65. C.H., H.E. and L.K. supervised the project.

Additional information
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