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SHORT COMMUNICATIONS

TubStain: a universal peptide-tool to label microtubules

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Abstract Imaging of the microtubular network is an important strategy to define cell-cycle specific and pathological states of eukaryotic cells. Here, we describe TubStain, a novel recombinant polypeptide allowing direct, non-antibody dependent labeling of microtubules in fixed cells over a broad range of different species and tissues. TubStain has, due to its small size and susceptibility for biochemical and genetic manipulations, high potential as a microtubule marker in state-of-the-art microscopy.

Keywords Cytochemistry · Immunohistochemistry · Fluorophore · Microtubules

Introduction

Microtubules are involved in many critical cellular processes, as mitotic spindle formation during cell division, construction of the cytoskeleton and shaping the cell architecture for cell migration and for intracellular transport of vesicles and organelles, as well as building the core structure of cilia and flagella (Janke and Bulinski 2011). Microtubules are organized as non-covalent polymers of alpha- and beta-tubulin heterodimers (Conde and Caceres 2009). They are highly dynamic as a result of constant polymerization and depolymerization. Usually, microtubules remain attached to the microtubule-organizing center by their less dynamic minus-end, while the well dynamic plus-end is unstable and grows outwards to the plasma membrane by polymerization of tubulin alpha/beta dimers. This extension and shortening of microtubules at the free ends, so called dynamic instability, is a major target for chemotherapeutic drugs against cancer (Risinger et al. 2009).

Due to their numerous dynamic duties, microtubules are highly attractive for various aspects of biological and biomedical research. Currently, different experimental approaches using fluorescence microscopy are used to visualize and investigate the organization and dynamics of microtubules. Expression or microinjection of recombinant tubulin subunits tagged with fluorophores is a powerful tool to study dynamics of the cytoskeleton in living cells (Kamath et al. 2010). Immunocytochemistry and immunohistochemistry using tubulin-specific poly- and monoclonal antibodies are the most common methods for labeling of the cytoskeleton in fixed cells and tissues. Detection of antibody–antigen complexes is mostly achieved by fluorophore-conjugated secondary antibodies. These indirect immunostaining procedures, however, require laborious, time- and cost consuming protocols and are limited by the number and quality of available primary antibodies which can be used within the same experiment.

To short-circuit these technical demands, we engineered a recombinant protein, called TubStain which allows versatile and direct labeling of microtubules in fixed cells over a broad variety of different species and tissues.

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Materials and methods

Expression and purification of TubStain

The conserved N-terminal domain of human PEX14 (microtubule binding domain, MTBD) was amplified by PCR (primers: 5′-AAGGATCCATGGCGTCCTCGGAGC AGGCAG-3′ and 5′-TTGTCGACTACGAAGGCTCATC GGCAGCAGTGC-3′ for PEX14(1-78), 5′-AAGGATCCG AAAATGTGCTGCCTCGAGAGC-3′ and 5′-TTGTCGA CTACGAAGGCTCATCGGCAGCAGTGC-3′ for PEX14 (20-78) and 5′-AAGGATCCATGGCGTCCTCGGAGCA GGCAG-3′ and 5′-TTTTGTCGACTAATCTGTCAGCCC TTTCTTCTTTAG-3′ for PEX14(1-60)) and cloned into Vector pGEX4T3 (GE Healthcare) using the *Bam*HI and SalI sites resulting in the core element of TubStain. Chemical labeling was performed according to the manufacturers protocols using thiol reactive (5-Iodoacetamidofluoresceine (5-IAF), Sigma; Dylight 488 maleimide, Thermo Fisher and tetramethylrhodamine-5-maleimide (TMR 5-maleimide), Invitrogen) and amine reactive (fluoresceine isothiocyanate (FITC), Invitrogen; Alexa 488 and 594 NHS esters, Invitrogen) dyes or colloidial gold (20 nm, Sigma). Fluorescent fusion proteins (XFPs: eGFP, eYFP, Venus,) were generated by cloning PCR product of XFP between GST and MTBD (primer pair: 5′-AAAGG ATCCATGGTGAGCAAGGGCGAGGAGC-3′ and 5′-AA AGGATCCCTTGTACAGCTCGTCCATGCCG-3′) using the *Bam*HI site or behind MTBD (primer pair: 5′-AAAGT CGACAATGGTGAGCAAGGGCG-3′ and 5′-AAAGCGG CCGCCTTGTACAGCTCGTCCATGC-3′) using SalI and NotI after changing the stop codon to Threonin (Quikchange, Quiagen; primer pair: 5′-CCGATGAGCCTTCGA CGTCGACTCGAGCGG-3′ and 5′-CCGCTCGAGTCGA CGTCGAAGGCTCATCGG-3′). *E. coli* optimized red fluorescent protein RFP_{EC} was generated by site-directed mutagenesis of *DsRed*-Express as reported previously (Pfleger et al. 2005) and subcloned as described for eGFP (primer pairs 5′-AAAGGATCCATGGCGAGCAGTGAG GACATCATCAAGGAGTTC-3′ and 5′-AAAGGATCCC AGGAACAGGTGGTGGCGG-3′ or 5′-AAAAGTCGACA ATGGCGAGCAGTGAGGAC-3′ and 5′-AAAGCGGCCG CCAGGAACAGGTGGTGGC-3′). Monomeric forms of GFP-derived XFPs were generated by introducing an A206K mutation (Quikchange, Quiagen; primer pairs: 5′-T ACCTGAGCTACCAGTCCAAGCTGAGCAAAGACCCC AAC-3′ and 5′-GTTGGGGTCTTTGCTCAGCTTGGACT GGTAGCTCAGGTA-3′ for eYFP and 5′-CCTGAGCACC CAGTCCAAGCTGAGCAAAGACCCCA-3′ and 5′-TGG GGTCTTTGCTCAGCTTGGACTGGGTGCTCAGG-3′ for eGFP) as described previously (Zacharias et al. 2002). Some XFPs were introduced using restriction free cloning as described previously (primer pairs 5′-ATGGTGAGCAA GGGCGAGGAGGATAACATGGCCATCATCAAGGAG TTCATGC-3′ and 5′-CTTGTACAGCTCGTCCATGCCG CCGGTGGAGTGGCGGCCCTCGGCG-3′ for RFPs or 5′-ATGGTGAGCAAGGGCGAGGAG-3′ and 5′-CTTGT ACAGCTCGTCCATGCCG-3′ for GFPs) (Unger et al. 2010). The 20 aa linker was introduced by cloning of the hybridized and 5′-phosphorylated primers 5′-GATCCG GTTCAGGCAGTGGTAGCGGTTCTGGATCCGGTACC GGTAGCGGAGGTACCGGAA-3′ and 5′-GATCTTCCG GTACCTCCGCTACCGGTACCGGATCCAGAACCGCT ACCACTGCCTGAACCG-3′ into the dephosphorylated *Bam*HI site.

Plasmids coding for TubStain (pGEX4T3, GE Healthcare) were transformed into BL21(DE3) cells. TubStain was expressed for 3 h at 30 °C in LB media containing 1 mM isopropyl-ß-D-thiogalactopyranosid. Cells were disrupted by ultrasonication (Digital Sonifier 250-D, Branson) and TubStain was purified using Protino® Glutathione Agarose 4B (Macherey–Nagel) according to the manufacturers protocol with PBS as buffer.

Cell cultures and tissues

Different cell lines and primary neuronal cell cultures were prepared as described previously: human fibroblast cell line GM5756T (Stanley et al. 2006), rat cerebellar slice cultures (Krah and Meller 1999), rat astrocytes (Theiss and Meller 2002), and dissociated chicken dorsal root ganglia neurons (Theiss et al. 2005). Formalin-fixed *Drosophila melanogaster* S2R+ cells were provided by Dr. Sven Bogdan (University Münster, Germany). Paraffin sections of chicken embryos were kindly provided by Prof. Dr. Beate Brand-Saberi and Dr. Verena Chankiewitz (Ruhr-University Bochum, Germany). For electron microscopy, human fibroblasts and N. ischiadicus of adult rats perfused with 2.5 % glutaraldehyd (GA) were used.

Immuno- and TubStaining

Cells on coverslips were fixed using 4 % paraformaldehyde for 10 min, washed 3 times (5 min each) with PBS, followed by membrane permeabilization using 0.5 % Triton X-100. After 3 times washing, blocking was performed for 30 min using 3 % BSA or goat serum (1:50) followed by incubation with TubStain (0.05 mg/ml) for various times (5 min to 4 h) and/or monoclonal tubulin antibody (Sigma T 4026). Using TubStain-GFP, optimal staining was achieved after 30 min incubation. Purkinje cells in rat cerebellar slice cultures were identified by anti-calbindin staining (Sigma, C 8666) and in some cells/tissues the actin-cytoskeleton was stained with phalloidin-TRITC (Sigma, P 1951). If antibodies were used, cells were washed 3 times and incubated with secondary antibody

(Sigma T 5393), then cells were washed 3 times including a DAPI stain (Sigma D 9542) and coverslips were mounted on glass slides using mounting medium (Dako, S3023).

Microscopy

Confocal images were taken with Zeiss LSM 510 and plan neofluar 40×/1.3 Oil or 63×/1.4 objectives, zoom level 1, 1,024 × 1,024 pixels, scan speed 6, average number 4. For imaging, the laser module contains a diode laser (405 nm), an Argon laser (488, 514 nm), and a Helium Neon laser (543 nm). Quantification of fluorescence intensity was done with aid of the physiology module (Zeiss LSM 510). The physiology module allowed to quantitatively monitor changes in fluorescence intensities in cells subsequent of drug treatment for various times with colchicine (10 μg/ml) or taxol (10 μg/ml).

After pre-embedding and labeling of microtubules with TubStain-Gold (20 nm), specimen for transmission-electron microscopy were fixed in 2.5 % GA, postfixed in OsO4, dehydrated and embedded in Epon. Sections were collected on Formvar-coated grids and contrasted with uranyl acetate. All samples were analyzed and recorded on a Philips EM 410 (Philips, Holland) transmission electron microscope equipped with a Gatan digital camera system at 80 kV voltage.

Results

Development and optimization of TubStain

Typically, TubStain consists of three modules: a microtubules-binding domain (MTBD), a dimerizer (recombinant protein glutathione-S-transferase GST) and a labeling agent (a fluorophoretic protein or dye or a gold particle) (Figs. 1, 2, 3). The MTBD is derived from PEX14, a central component of the peroxisomal protein import machinery. The in vitro binding capacity of GST-PEX14(1-78) and β-tubulin has recently been demonstrated (Bharti et al. 2011). As a first step in the development of TubStain, we tested the GST-tagged MTBD for indirect labeling of microtubules in PFA-fixed fibroblasts. For this purpose, we incubated fixed and solubilized cells with purified GST-tagged MTBD and monitored its subcellular localization by immunofluorescence microscopy with anti-GST- or anti-PEX14 antibodies. This procedure clearly labeled microtubules or microtubules and peroxisomes, respectively (data not shown). No efficient labeling of microtubules was achieved when monomeric His-tagged MTBD was used at the same concentration followed by detection using anti-PEX14 antibodies (data not shown) suggesting that dimerization via GST improves the sensitivity.

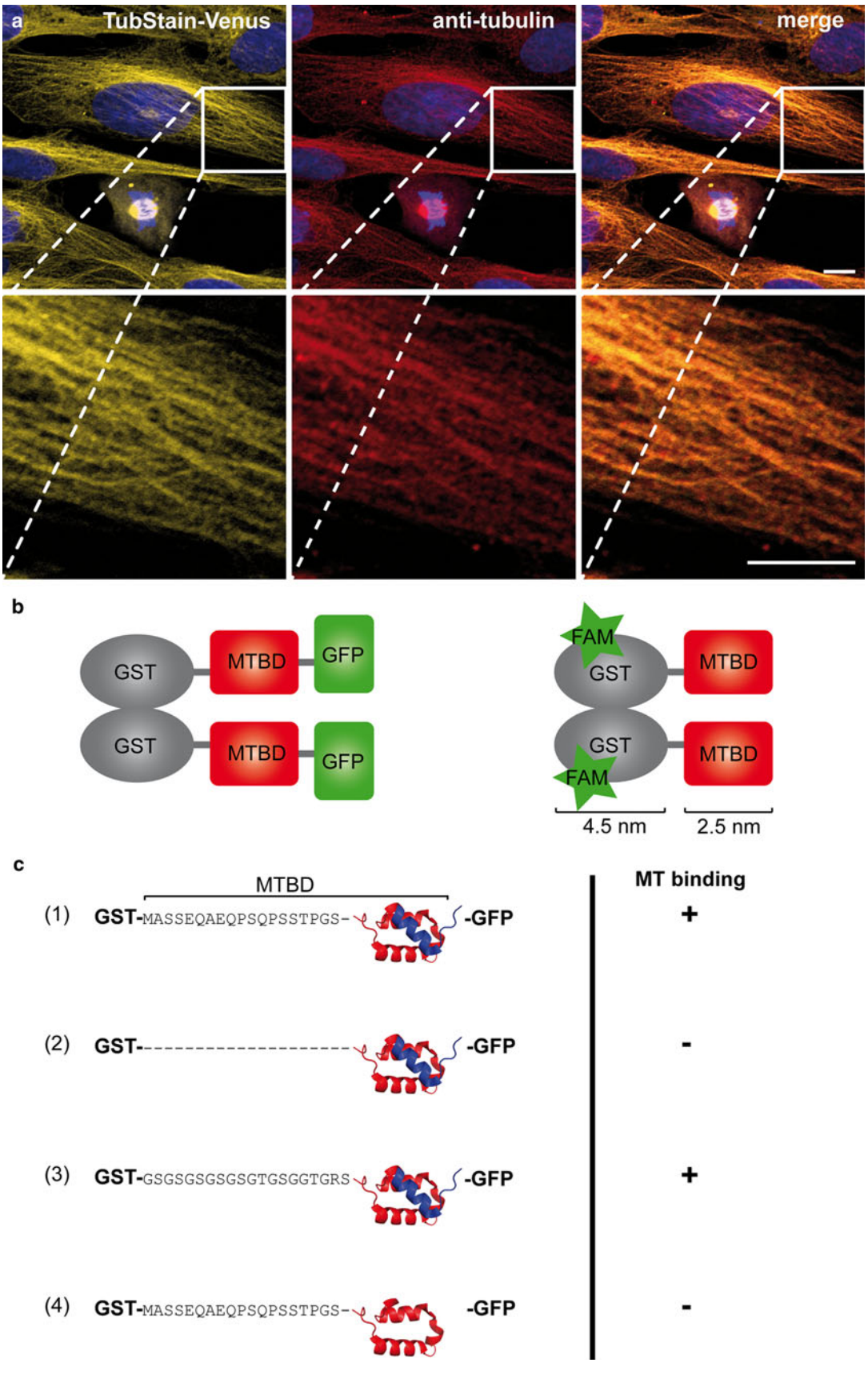
We took advantage of the ability of GST-tagged MTBD to label microtubules and developed a first version of TubStain by fusing GST-tagged MTBD to a fluorescent protein for direct in vitro labeling of microtubules in fixed fibroblasts. Co-immunostaining with anti-β-tubulin antibodies revealed perfect overlap in fluorescence microscopy (Fig. 1a). Again no efficient labeling of microtubules was achieved when monomeric His-tagged MTBD was used at the same concentration (data not shown).

However, when the fluorescent protein was inserted between GST and MTBD, the oligomerization properties of the GFP variant became critical. A construct containing the strict monomeric GFP variant GFP(A206K) at this position could be successfully used as a microtubular marker whereas constructs containing GFPs tending to dimerize were not suitable (data not shown). These observations support the conclusion that the steric distance between the two MTBD is critical for the affinity of TubStain.

Next, we analyzed which properties of linker regions between different TubStain domains are critical for labeling of microtubules. To this end, we tested various MTBD variants, all based on the prototypic GST-MTBD-GFP (Fig. 1b, c). The MTBD consists of Pex14(1-78) which is characterized by (1) a 20 amino acids N-terminal extension which is not conserved and (2) a very well conserved core-region of amino acids 21–78 which mediates interactions with other peroxisomal membrane proteins. Whereas the N-terminal stretch seems to be intrinsically unstructured, the core-region folds into a bundle of three alpha helices (Neufeld et al. 2009; Su et al. 2009). Removal of the N-terminal extension abolished microtubule binding capacity. In contrast, substitution of first 20 amino acids of PEX14(1-78) by a 20 amino acid serine-glycine-linker did not affect labeling of microtubules. This suggests that the amino acid sequence of the N-terminal extension does not significantly contribute to microtubule binding but the presence of such a stretch. We also tried to shorten the alpha-helical core region by removing the stabilizing C-terminal helix. However, no labeling of microtubules was achieved with such a truncated TubStain, suggesting that the MTBD requires the compact fold for efficient MT binding.

Various fluorophore labeling of TubStain

The next step aimed to create a variety of fluorescent TubStain derivatives based on the dimeric MTBD that are suitable for fluorescence microscopy. Both the position within the construct and the oligomeric properties of fluorescent proteins were critical for specificity of labeling. Various C-terminal fusions with fluorescent proteins XFP


a
TubStain-Venus
anti-tubulin
merge
b
GST
MTBD
GFP
GST
MTBD
GFP
FAM
GST
MTBD
GST
MTBD
FAM
4.5 nm
2.5 nm
c
MTBD
MT binding
(1) GST-MASSEQAEQPSQPSSTPGS- -GFP
+
(2) GST- -GFP
-
(3) GST-GSGSGSGSGSGTGSGGTGRS -GFP
+
(4) GST-MASSEQAEQPSQPSSTPGS- -GFP
-

◀ **Fig. 1** Biochemical characterization and application of TubStain. **a** Labeling of microtubules by TubStain and anti-tubulin antibodies in paraformaldehyde fixed human fibroblasts. Confocal laser scanning microscopy of TubStain-Venus and anti-Tubulin antibodies reveal co-staining of the microtubules network (merge). *Scale bars* 10 μm. **b** TubStain design with various labels like fluorescent fusion proteins (e.g. GFP) or fluorophoretic dyes, e.g. fluoresceine (FAM). The glutathion-S-transferase (GST) and the microtubule binding domain (MTBD) modules are indicated. **c** Effects of truncations and amino acid substitutions of the MTBD on microtubule binding capacity. TubStain containing the MTBD PEX14(1-78) is able to label microtubules (1), while TubStain containing amino acids 20–78 of PEX14 does not bind microtubules (2). Insertion of a 20 amino acid linker between GST and PEX14(20-78) restores microtubule binding capacity (3). Deletion of amino acids 61–78 depletes microtubule binding (4)

(eGFP, Venus, eYFP, and RFP_{EC}) specifically labeled microtubules (Figs. 1b, 2a–d).

A clear labeling of MT was also seen with GST-MTBD conjugated with non-proteinaceous fluorescent dyes as the cysteine-labeling dyes 5-IAF (Figs. 1b, 2e) as well as Alexa Fluor 488 (Fig. 2f), respectively. In contrast, attachment of the lysine-labeling dye Alexa 488 NHS considerably decreased microtubule-binding capacity of GST-MTBD (data not shown), suggesting that one or more lysine residues within MBD are required for its function as microtubule marker. As MTBD is a recombinant protein, desired properties might be introduced by site-directed mutagenesis. Thus, it is for example possible to introduce a single cysteine to conjugate a fluorophore close to the binding site for tubulin. The possibility of dye-labeling of TubStain certainly has a great potential in the new area of high-resolution microscopy since it allows to place the dye much closer to its target than indirect labeling techniques, which often result in non-optimal resolution (Fernandez-Suarez and Ting 2008).

Gold particles conjugated to TubStain

Electron microscopy is a powerful tool to investigate the assembly, organization and distribution of microtubules with a resolution superior to light microscopy, including confocal laser scanning microscopy. To further prove the versatility of TubStain, the GST-MTBD module was coupled to 20 nm gold particles (TubStain-Gold) for the use in electron microscopy. TubStain-Gold was used in glutaraldehyde fixed human fibroblasts in vitro (Fig. 3a, b) and rat N. ischiadicus (Fig. 3c) to directly label microtubules. Remarkably, the gold particles are directly located or closely adjacent to the microtubules, which is due to the very small spatial extent of 7 nm of TubStain. Therefore, TubStain-Gold is a suitable and convenient microtubule marker for electron microscopy.

TubStain as a quantitative tool

In clinical use anticancer therapy is often based on drugs affecting microtubule assembly and microtubule dynamics, and finally acting as antimitotic drugs. Although most microtubule-interfering agents either stabilize or destabilize tubulin via binding to known tubulin-binding sites, some compounds bind to tubulin at undefined sites (Stanton et al. 2011). Therefore, visualization of microtubular networks is often a critical step of screening and characterization of microtubules-based drugs, which have a high potential in cancer treatment. Here, a direct labeling agent like TubStain might be advantageous over immunostaining methods. Thus, we performed studies using human fibroblasts treated with colchicine or taxol for 4 and 8 h to check if depolymerization or aggregation of microtubules can be measured directly with the aid of TubStain (Fig. 4). Colchicine was the first identified antimitotic agent which suppresses assembly dynamics of microtubule ends. At high concentrations, it induces microtubule-depolymerization by binding to the intradimeric alpha–beta interface of tubulin heterodimes (Ravelli et al. 2004; Risinger et al. 2009). In contrast, taxol (paclitaxel), an alkaloid of the Pacific yew Taxus revifolia, is known to promote polymerization of microtubules (Schiff and Horwitz 1980). Furthermore, taxol almost blocks the dissociation of tubulin heterodimers (Parekh and Simpkins 1997), and causes an accumulation of bundles of microtubules (Theiss and Meller 2000; Giessmann et al. 2005).

Quantification of TubStain-fluorescence of cells treated with either colchicine or taxol for various times (4, 8 h) using Zeiss physiology software revealed that TubStain is a suitable tool to study polymerizing/depolymerizing drugs (Fig. 4). A decreased fluorescence intensity was detected after colchicine incubation, whereas taxol led to an increase in fluorescence intensity within the cells, which is directly related to the depolymerization/polymerization of microtubules. Therefore, quantification by direct TubStaining is more reliable than immunostaining which often suffers from amplification effects caused either by indirect detection by secondary antibodies or by a varying number of fluorophores attached to each antibody.

TubStain labeling in various species and tissues

Microtubules show an ubiquitous distribution in eukaryotes with numerous functions in different cell types. They constitute the mechanical cytoskeleton of cells, determining the cell morphology, provide the framework for the intracellular transport, built the mitotic spindle, and moreover they are the major framework of cilia and flagella. Therefore, we tested the labeling capacity of TubStain in a variety of species and organs in vivo and in vitro.

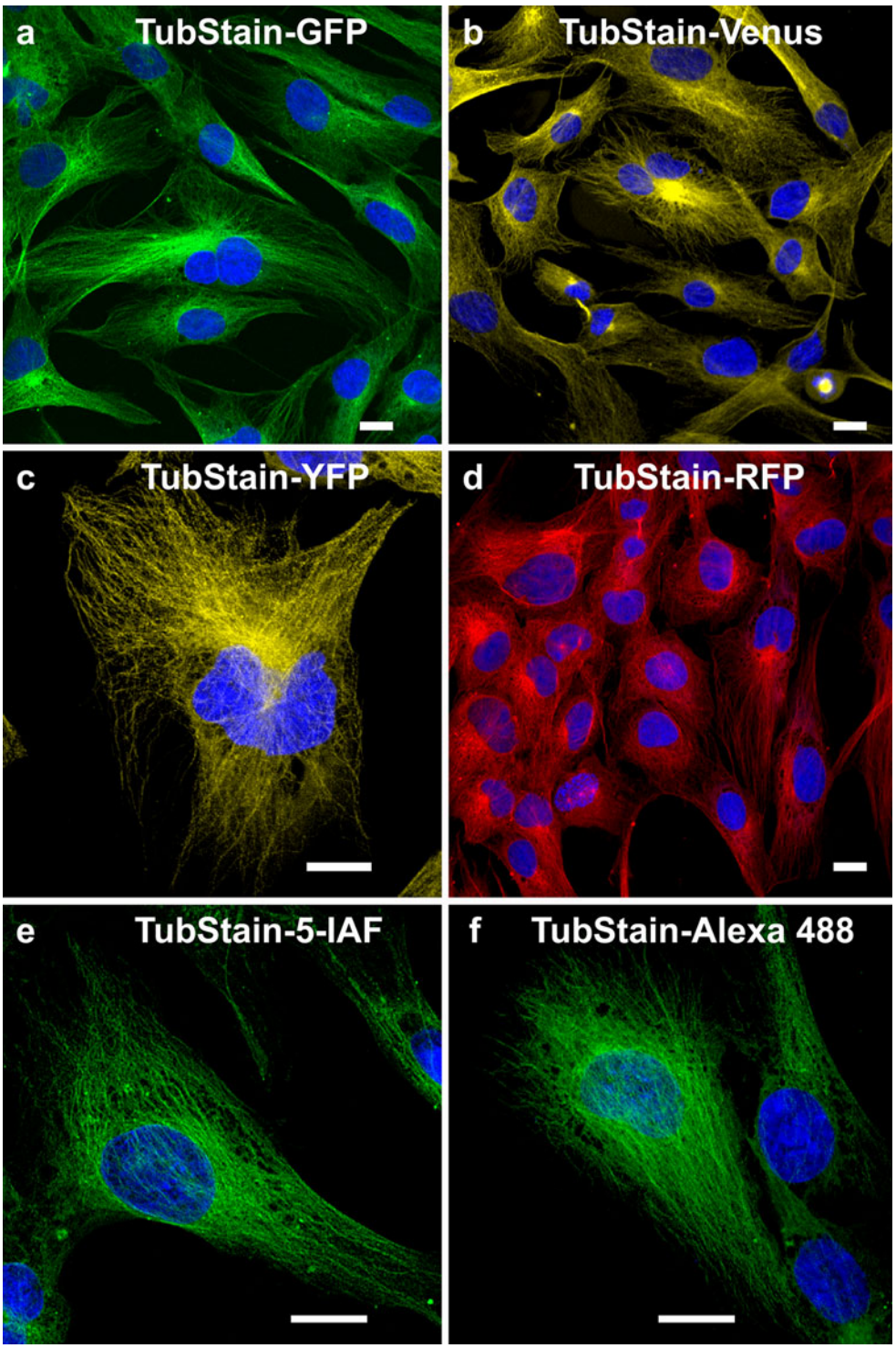


Fig. 2 Staining of microtubules with fluorescent variants of TubStain. Confocal laser scanning microscopy of TubStain-labelled microtubules in fixed human fibroblasts and nuclear counterstain with DAPI. **a** TubStain-GFP, **b** TubStain-Venus, **c** TubStain-YFP, **d** TubStain-RFP_{EC}, **e** TubStain-5-IAF (5-Iodoacetamidofluorescein), **f** TubStain-Alexa 488 (AlexaFluore 488 C5 maleimide). *Scale bars* 20 μm

We also checked the feasibility of TubStain to identify microtubules with different histological protocols, like for example in whole mount biologics or after paraffin sectioning. It is remarkable that TubStain labeled microtubules in human fibroblasts, mammalian and chicken nervous tissue and also in insects (Fig. 5). TubStain proved to be suitable for histochemical labeling of paraffin-sections of neural tubes of chicken embryos. In all these cells and

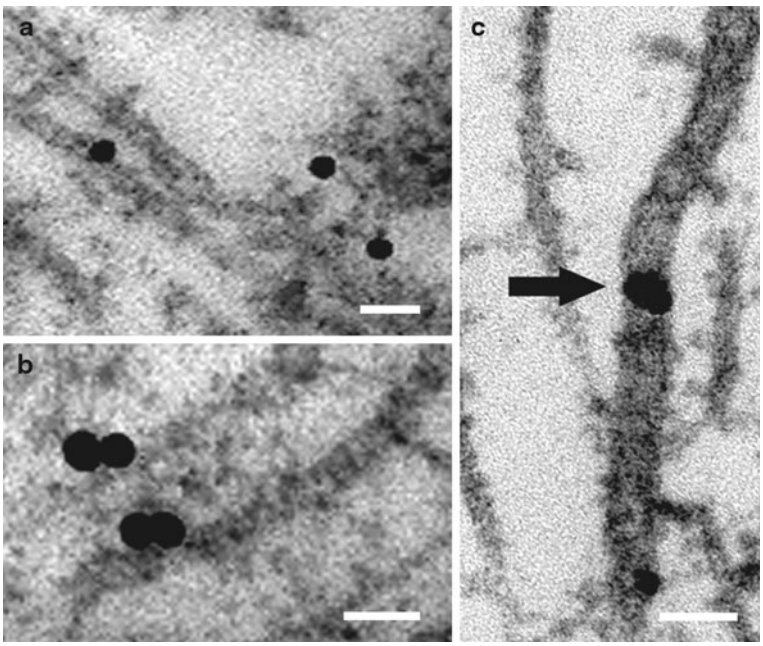


Fig. 3 Application of TubStain-Gold for electron microscopy. Ultra-structural labeling of microtubules with TubStain-Gold (20 nm) in human fibroblasts (**a**, **b**) and in rat Nervus ischiadicus (**c**). *Scale bars* 50 nm

tissues, TubStain revealed a clear labeling of microtubules, comparable to the staining with anti-tubulin antibodies. In these tissues, TubStain can be combined with nuclear Hoechst staining, certain antibody protocols as well as phalloidin-labeling. In conclusion, TubStain is a novel universal tool for labeling of microtubules of paraformaldeyde and glutaraldehyde fixed cells.

Discussion

TubStain is a novel peptidic marker engineered for labeling of microtubules of fixed cells. The affinity and specificity of TubStain is provided by a microtubule-binding domain (MTBD) which is a derivative of the conserved N-terminal domain of PEX14, a peroxisomal membrane protein with multiple tasks in biogenesis (Meinecke et al. 2010; Neufeld et al. 2009), motility (Bharti et al. 2011) and degradation of these organelles (Farre et al. 2009; Zutphen et al. 2008; Hara-Kuge and Fujiki 2008). TubStain consist of three modules, the PEX14 derived MTBD, a dimerizer (here GST) and a fluorophore which might be a fluorescent protein or dye. The GST-module allows the one-step affinity purification of the recombinant TubStain. In addition, it is critical for microtubule binding. Accordingly, MTBD without GST does not stain microtubules or interact with purified tubulin in vitro (data not shown). Moreover, insertion of bulky proteins like oligomeric GFP variants between MTBD and the GST moiety impairs binding to microtubules. Together, these observations imply that dimerization of MTBD is a prerequisite to bind polymeric tubulin.

As MTBD is derived from PEX14, monomer–dimer transitions provide a possible explanation for the observed functional heterogeneity of PEX14. In vitro data suggest that dimeric PEX14 dissociates into monomers after binding of peroxisomal protein import receptors (Su et al. 2010). In vivo data show that various oligomeric states of PEX14 exist at the peroxisomal membrane (Itoh and Fujiki 2006). Based on our data we suggest that the dimeric form of PEX14 binds to microtubules in vivo.

Another critical factor in the design of TubStain is the conjugation of dyes or gold-particles to GST-MTBD without affecting the microtubule affinity or selectivity in various microscopical techniques. Unspecific amine-coupling of non-proteinaceous fluorescent dyes is not applicable since several lysines of the MTBD are required for interaction with tubulin. Cysteine modifications are suitable since the MTBD does not contain such residues. Thereby, the four cysteine residues of GST were labeled by the fluorophoretic dye 5-IAF or gold-particles. While the TubStain-coupled gold-particles seem to bind microtubules specifically, some background staining could be observed by confocal laser scanning microscopy. The best results for TubStaining in fluorescence microscopy without any background staining were achieved when GST-MTBD was directly fused at its C-terminus to fluorophoretic proteins eGFP, Venus, eYFP, and RFP_{EC}. The purified recombinant proteins are stable over months and have several advantages in comparison to the commonly used anti-tubulin antibodies. First, TubStain is a direct labeling agent of microtubules. Indirect immunostaining is more time-consuming, cost-intensive and relies on species-specific secondary antibodies. Second, TubStain is a universal

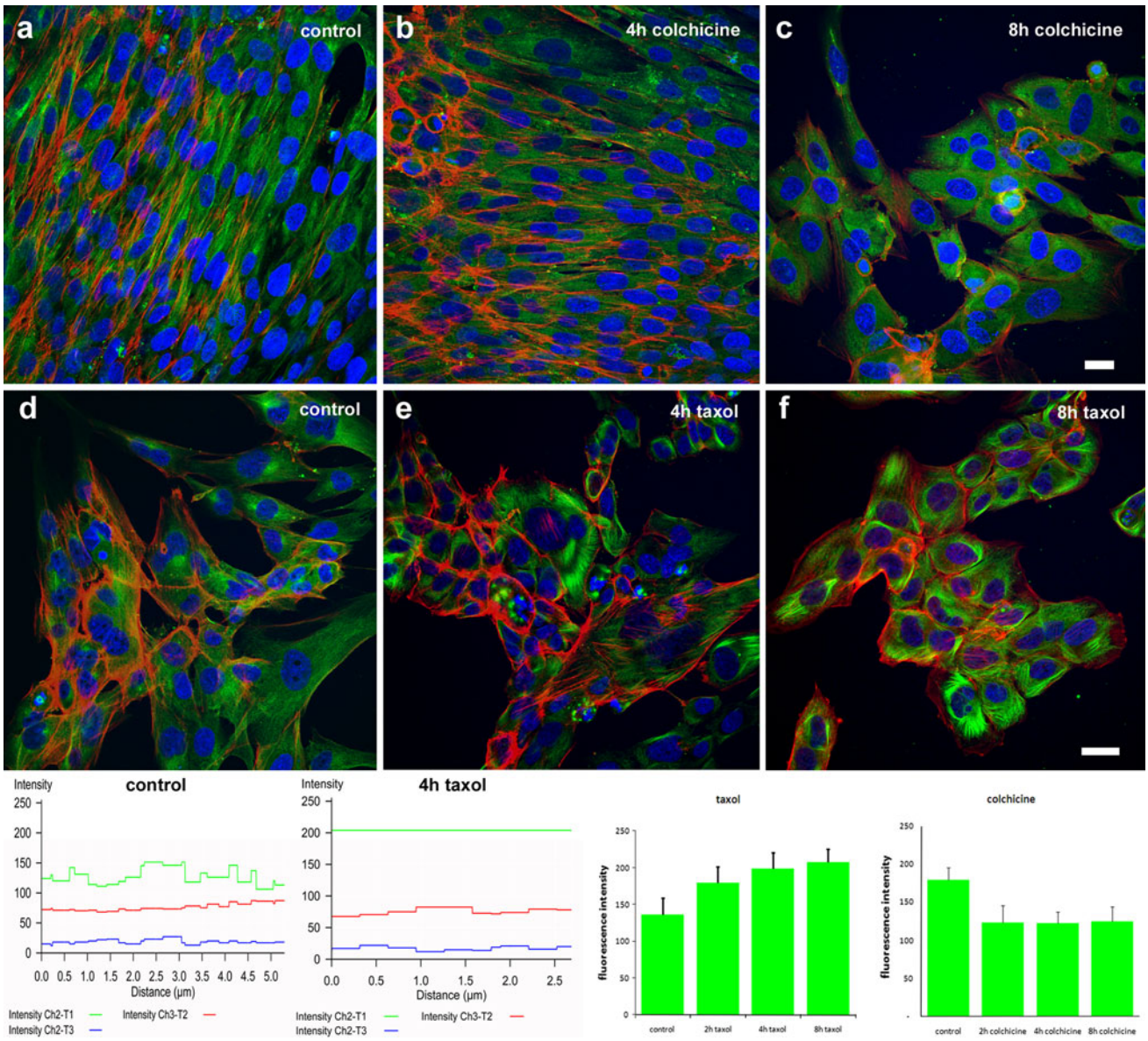


Fig. 4 Visualization of microtubule depolymerization or bundling. Confocal laser scanning microscopy of TubStain in human fibroblasts treated with either colchicine (**a**–**c**) or taxol (**d**–**f**). With time incubation leads to depolymerization (colchicine) or bundling (taxol) of the microtubules. This affects can be directly visualized by TubStain. With aid of the appropriate imaging software the fluorescence intensity of TubStain-GFP in controls and treated cells is directly correlated with depolymerization (**a**–**c**) or bundling (**d**–**f**) of microtubules (*lower panels*). Fibroblasts were counterstained with phalloidin-TRITC and DAPI. *Scale bar* 20 µm

labeling tool in a broad variety of tissues and cells whereas many antibodies are restricted to certain species. Third, due to the small size of TubStain, it is particularly suitable for high-resolution microscopy applications. It is well known that indirect immunolabeling techniques often result in non-optimal resolution (Fernandez-Suarez and Ting 2008). TubStain has a high susceptibility to genetic manipulation, thereby allowing to incorporate suitable fluorophoretic dyes in a site-specific manner close to the tubulin binding site. Fourth, fluorescence quantification by direct TubStaining is more reliable than immunostaining which often suffers from amplification effects caused either by indirect detection by secondary antibodies or by a varying number of fluorophores attached to each antibody. Visualization of microtubular networks is often a critical step of screening and characterization of microtubules-based drugs which have a high potential in cancer treatment (Risinger et al. 2009).

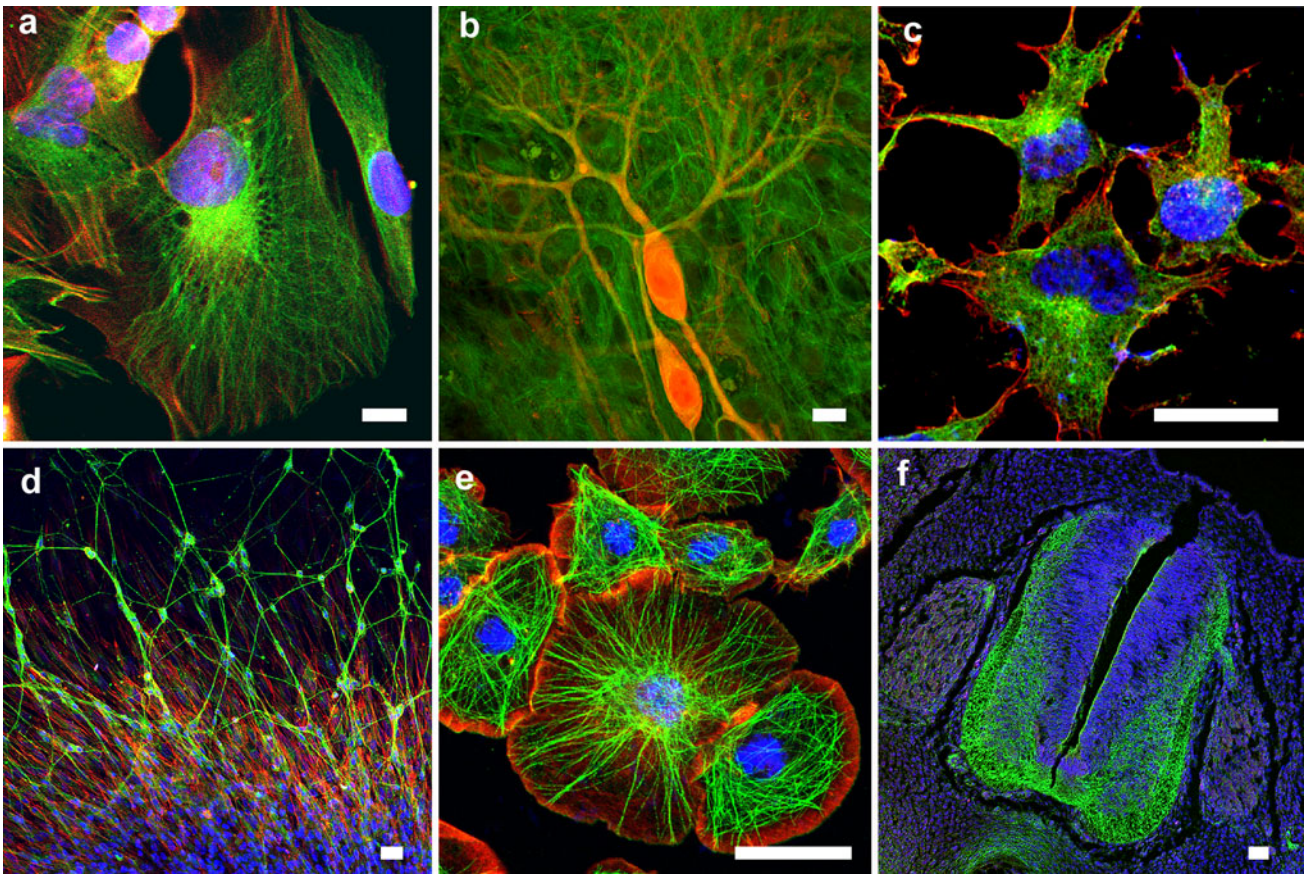


Fig. 5 Confocal laser scanning microscopy of TubStain in various cells, tissues and species in vitro (**a**–**e**) and in vivo (**f**–**i**). Staining of microtubules with TubStain-GFP (**a**) in human fibroblasts (counterstained with phalloidin-TRITC, DAPI), (**b**) in Purkinje cells of rat cerebellar slice cultures (counterstained with anti-calbindin (TRITC)), (**c**) cultured rat astrocytes (counterstained with phalloidin-TRITC, DAPI), (**d**) outgrowing neurites of chicken dorsal root ganglia (counterstained with phalloidin-TRITC, DAPI), (**e**) fruit fly S2R+ cells (counterstained with phalloidin-TRITC, DAPI), (**f**) neural tube (chicken embryo, HH27, paraffin section, TubStain-GFP, DAPI). *Scale bars* 20 μm

Finally, TubStain is powerful in combination with other cellular markers as phalloidin, mito-tracker as well as in combination with all kinds of antibody labeling.

In summary, TubStain offers good prospects as alternative to commonly used microtubule antibodies in immunohistochemical and other microscopy applications in the whole area of cell biological, medical and pharmaceutical research.

References

Bharti P, Schliebs W, Schievelbusch T, Neuhaus A, David C, Kock K, Herrmann C, Meyer HE, Wiese S, Warscheid B, Theiss C, Erdmann R (2011) PEX14 is required for microtubule-based peroxisome motility in human cells. J Cell Sci 124(Pt 10): 1759–1768

Conde C, Caceres A (2009) Microtubule assembly, organization and dynamics in axons and dendrites. Nat Rev Neurosci 10(5): 319–332

Farre JC, Krick R, Subramani S, Thumm M (2009) Turnover of organelles by autophagy in yeast. Curr Opin Cell Biol 21(4):522–530

Fernandez-Suarez M, Ting AY (2008) Fluorescent probes for super-resolution imaging in living cells. Nat Rev Mol Cell Biol 9(12):929–943

Giessmann D, Theiss C, Breipohl W, Meller K (2005) Decreased gap junctional communication in neurobiotin microinjected lens epithelial cells after taxol treatment. Anat Embryol (Berl) 209(5):391–400

Hara-Kuge S, Fujiki Y (2008) The peroxin Pex14p is involved in LC3-dependent degradation of mammalian peroxisomes. Exp Cell Res 314(19):3531–3541

Itoh R, Fujiki Y (2006) Functional domains and dynamic assembly of the peroxin Pex14p, the entry site of matrix proteins. J Biol Chem 281(15):10196–10205

Janke C, Bulinski JC (2011) Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions. Nat Rev Mol Cell Biol 12(12):773–786

Kamath K, Oroudjev E, Jordan MA (2010) Determination of microtubule dynamic instability in living cells. Methods Cell Biol 97:1–14

Krah K, Meller K (1999) Axonal and dendritic transport in Purkinje cells of cerebellar slice cultures studied by microinjection of horseradish peroxidase. Cell Tissue Res 295(1):55–64

Meinecke M, Cizmowski C, Schliebs W, Kruger V, Beck S, Wagner R, Erdmann R (2010) The peroxisomal importomer constitutes a large and highly dynamic pore. Nat Cell Biol 12(3):273–277

Neufeld C, Filipp FV, Simon B, Neuhaus A, Schuller N, David C, Kooshapur H, Madl T, Erdmann R, Schliebs W, Wilmanns M, Sattler M (2009) Structural basis for competitive interactions of Pex14 with the import receptors Pex5 and Pex19. EMBO J 28(6):745–754

Parekh H, Simpkins H (1997) The transport and binding of taxol. Gen Pharmacol 29(2):167–172

Pfleger BF, Fawzi NJ, Keasling JD (2005) Optimization of DsRed production in Escherichia coli: effect of ribosome binding site sequestration on translation efficiency. Biotechnol Bioeng 92(5):553–558

Ravelli RB, Gigant B, Curmi PA, Jourdain I, Lachkar S, Sobel A, Knossow M (2004) Insight into tubulin regulation from a complex with colchicine and a stathmin-like domain. Nature 428(6979):198–202

Risinger AL, Giles FJ, Mooberry SL (2009) Microtubule dynamics as a target in oncology. Cancer Treat Rev 35(3):255–261

Schiff PB, Horwitz SB (1980) Taxol stabilizes microtubules in mouse fibroblast cells. Proc Natl Acad Sci USA 77(3):1561–1565

Stanley WA, Filipp FV, Kursula P, Schuller N, Erdmann R, Schliebs W, Sattler M, Wilmanns M (2006) Recognition of a functional peroxisome type 1 target by the dynamic import receptor Pex5p. Mol Cell 24(5):653–663

Stanton RA, Gernert KM, Nettles JH, Aneja R (2011) Drugs that target dynamic microtubules: a new molecular perspective. Med Res Rev 31(3):443–481

Su JR, Takeda K, Tamura S, Fujiki Y, Miki K (2009) Crystal structure of the conserved N-terminal domain of the peroxisomal matrix protein import receptor, Pex14p. Proc Natl Acad Sci USA 106(2):417–421

Su JR, Takeda K, Tamura S, Fujiki Y, Miki K (2010) Monomer-dimer transition of the conserved N-terminal domain of the mammalian peroxisomal matrix protein import receptor, Pex14p. Biochem Biophys Res Commun 394(1):217–221

Theiss C, Meller K (2000) Taxol impairs anterograde axonal transport of microinjected horseradish peroxidase in dorsal root ganglia neurons in vitro. Cell Tissue Res 299(2):213–224

Theiss C, Meller K (2002) Microinjected anti-actin antibodies decrease gap junctional intercellular commmunication in cultured astrocytes. Exp Cell Res 281(2):197–204

Theiss C, Napirei M, Meller K (2005) Impairment of anterograde and retrograde neurofilament transport after anti-kinesin and anti-dynein antibody microinjection in chicken dorsal root ganglia. Eur J Cell Biol 84(1):29–43

Unger T, Jacobovitch Y, Dantes A, Bernheim R, Peleg Y (2010) Applications of the Restriction Free (RF) cloning procedure for molecular manipulations and protein expression. J Struct Biol 172(1):34–44

Zacharias DA, Violin JD, Newton AC, Tsien RY (2002) Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. Science 296(5569):913–916

Zutphen T, Veenhuis M, van der Klei IJ (2008) Pex14 is the sole component of the peroxisomal translocon that is required for pexophagy. Autophagy 4(1):63–66