

Subproject E1.5

Use of Nanoparticles to Study and Manipulate the Polarity of Plant Cells

Principle Investigator: Peter Nick

CFN-Financed Scientists: K. Eggenberger (1/2 BATIIa/E13, 33 months), N. Frey (1/2 E13, 9 months), P. Hohenberger (BATIIa, 7 months), O. Iwanowa/Sosedowa (1/2 BATIIa, 15 months), A. Jovanovic (1/2 E13, 29 months), M. Ouko (1/2 E13, 5 months), I. Njimona (1/2 E13, 2.5 months)

Further Scientists: N. Kiknadze, J. Kobrlová, K. Schwarzerová, N. Hayashi, Ute Schepers

**Botanisches Institut 1
Universität Karlsruhe (TH)**

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Introduction and Summary

The goal of project E1.5 is to develop tools for the application of nanoparticles to plant-cell biology. To achieve this goal, three tasks have to be solved: 1. The nanoparticles have to be rendered biocompatible such that they are not toxic, soluble in aqueous solution, and amenable to conjugation with proteins of interest. 2. The nanoparticles have to be introduced into the cell, which is a special challenge in plant cells that are surrounded by a cellulosic cell wall. 3. The nanoparticles have to be adapted for visualization and manipulation in the context of cell-biological applications.

1. Bioconjugation

Different strategies of bioconjugation were tested using monoclonal antibodies directed against tubulin, we were able, for the first time, to visualize plant microtubules *in situ* by luminescent CdSe semiconductor nanoparticles. Functionalized silica-shells were found to be superior to BSA-ligand exchange for cell biological applications with respect to signal preservation of cellular target (microtubules) [E1.5:10, E1.5:12, E1.5:24].

2. Introduction into plant cells

Several physical approaches have been tested and improved such as particle bombardment [E1.5:3, E1.5:8, E1.5:18], microinjection or nanopulse electrical discharge [E1.5:22]. However, we focussed, in cooperation with the groups of Stefan Bräse and Anne Ulrich, chemical approaches of membrane passage:

We were able to define **cell-permeating peptides** that leave the vacuolar membrane intact, which allowed to label actin filaments by phalloidin in living cells. However, pore sizes were too small for the introduction of peptides or other nanosized cargoes [E1.5:24]. By using the synthetic peptide BP100 in fusion with the actin-binding peptide Lifeact, we could recently label the actin cytoskeleton of living plant cells by chemical engineering without genetic transformation [E1.5:26].

In parallel, we explored **Trojan Peptoids** for the uptake into plant cells. We could show that fluorescently labelled polyguanidine peptoids enter rapidly into tobacco BY-2 cells without affecting viability. The uptake is fast, independent of receptor-mediated endocytosis, but involves both microtubules and actin filaments [E1.5:12, E1.5:20]. A third-generation derivative of these carriers could be specifically targeted to plant mitochondria. This allowed, for the first time, to visualize the movement of mitochondria along actin filaments by high-resolution 4D confocal microscopy [E1.5:25] yielding high-quality information on individual trajectories that could be rendered and quantified in great detail using the IMARIS image analysis package leading to novel insights into the role of actin polymerization as driving force of mitochondrial movement.

3. Adaptation of nanoparticles for plant-cell biology

The motivation for this project is the manipulation of plant cells by introducing nano-sized cargoes with specific properties. We have explored this along two lines: by adapting the nanosized cargo and by improving the specificity of the molecular transporter.

Adapting nanosized cargoes: We showed that CdSe nanocrystals can be used for direct immunofluorescence of plant microtubules *in situ*, making use of their superior optical

properties [E1.5:10, E1.5:12, E1.5:24], which allows to circumvent cross-reactions between different monoclonal antibodies. We explored the potential toxicity of leaking Cd^{2+} ions, and could show that Cd can induce programmed cell death depending on the phase of the cell cycle [E1.5:13, E1.5:20]. We explore less toxic alternatives (ZrS, in cooperation with the group of C. Feldmann or InP, in cooperation with the group of T. Nann, Norwich). In cooperation with the group of L. Fruk, we have tested bio-functionalized TiO_2 nanoparticles, and successfully induced plant apoptosis by a short pulse of UV-light (Krahmer, Fruk, and Nick, unpublished).

Improving the specificity of molecular transporters: In cooperation with the groups Bräse, Schepers, and Balaban, mitochondria-targeted Trojan Peptoids have been functionalized with a light-activable chromophore and introduced into mammalian and plant cells [E1.5:25] to induce apoptosis by a light pulse. In cooperation with the group of A. Ulrich, the tool-kit for chemical engineering is extended by functional screening of new structures, and fusion with specific peptides that compete with innate proteins for their natural binding site using actin nucleation as proof-of-principle system.

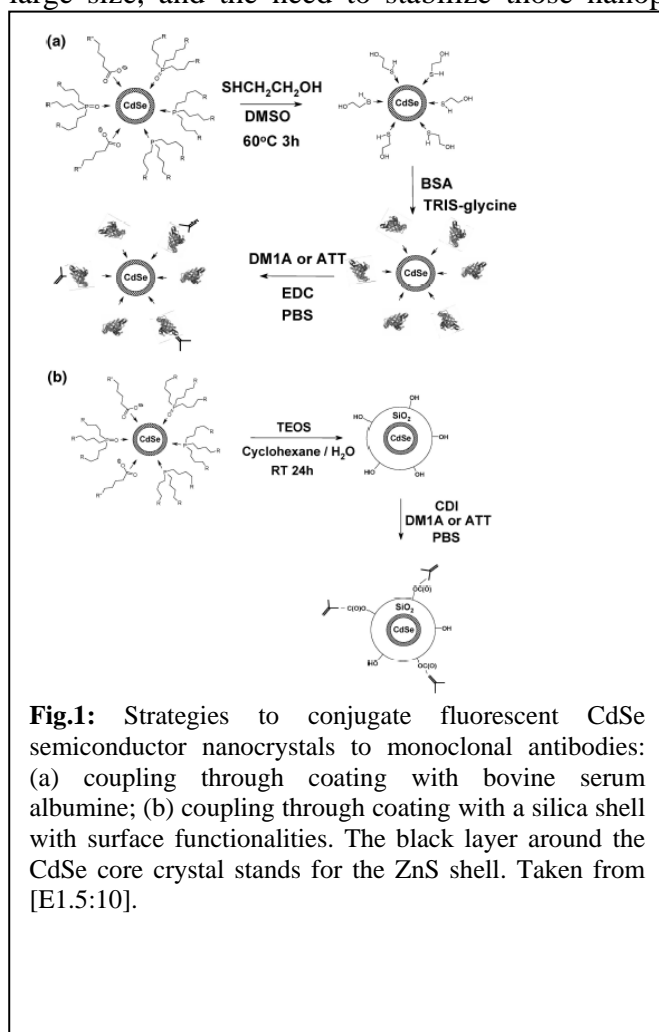
1. Bioconjugation

Luminescent semiconductor nanocrystals are of great interest for cell biology, because of their increased fluorescence intensity and bleaching resistance. In addition, they can be excited over a broad wavelength range but emit with a narrow band that can be tuned by the size of the particles. However, the application of these nanocrystals for cell biology is limited by their cytotoxicity, their large size, and the need to stabilize those nanoparticles colloiddally in the aqueous environment

typical for biological applications. To overcome these limitations, we have tested two strategies to coat CdSe semiconductor nanocrystals (**Fig. 1**):

1. Coating with BSA through ligand exchange (**Fig. 1a**). This approach provides a better electrostatic colloidal stabilization of the nanoparticles in water and is more versatile with respect to active groups for the covalent coupling of proteins. Moreover, the resulting particles are smaller in diameter.

2. Coating with silica shells (**Fig. 1b**). The silanol groups on the surface decrease hydrophobicity and the tendency to agglomerate in aqueous environment. Silica is inert in most solvents, which should reduce any noxious effects on the cells. Silica coats are tighter against leakage of the toxic Cd^{2+} , which is important to prevent Cd-induced programmed cell death [E1.5:13]. It is possible to introduce specific surface functionalities by modification of surface hydroxyls with amines, thiols, carboxyls, or methacrylate.



To date, there is no general solution for coupling biomolecules to nanoparticles under preservation of their biological activity. To assess the feasibility of the two bioconjugation strategies, we used monoclonal antibodies directed against α -tubulin as target protein and used visualization of plant microtubules *in situ* by direct immunofluorescence with these nanocrystal-conjugated antibodies as readout. Both nanoparticle-antibody conjugates were used to follow the dynamic reorganization of microtubules through the cell cycle of a tobacco cell culture in double and triple staining with fluorescein-isothiocyanate as conventional fluorochrome and Hoechst 33258 as marker for mitotic duplication of DNA. BSA-coated nanocrystals visualized fluorescent dots that decorated the various arrays of microtubules. The specificity of the antibody was maintained after conjugation with the nanocrystals, and the antibodies correctly represented the dynamics of cell-cycle-dependent microtubular reorganization (**Fig. 2**, left). However, this approach did not yield a contiguous signal. In contrast, silica-shelled nanocrystals visualized contiguous microtubules (**Fig. 2**, right) in the same pattern as found for the conventional fluorochrome FITC (**Fig. 2**, center) and thus can be used as labels for direct immunofluorescence in plant cells. Given the fact that up to 50 secondary antibodies bind to one primary antibody in the conventional indirect immunofluorescence (**Fig. 2**, center), one has to conclude that the fluorescence intensity produced by an individual nanocrystal-conjugated antibody is very high. The use of indirect immunofluorescence is strongly limited in multiplexing, where several signals have to be detected simultaneously. It is extremely difficult to safeguard against illegitimate cross-reaction of the secondary fluorescent antisera. Direct immunofluorescence would be a good alternative but has been limited by low signal strength because the signal amplification due to the labeled secondary antibody is lacking. We could demonstrate in our study that this limitation can be overcome by direct coupling of fluorescent CdSe semiconductor nanocrystals to highly specific antisera.

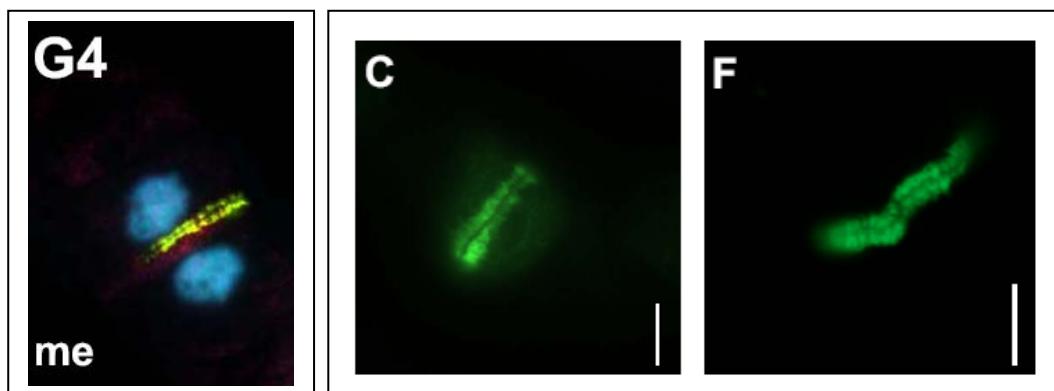


Fig.2: Direct immunofluorescence of the microtubular phragmoplast array in a telophasic tobacco BY-2 cell with monoclonal mouse anti-tubulin antibodies conjugated to BSA-coated CdSe nanocrystals (left) or with silica-shelled nanocrystals (right). The signal for conventional indirect immunofluorescence is shown in the center. Taken from [E1.5:10].

2. Introduction into plant cells

To extend the potential of nanoparticles from *in-vitro* and *in-situ* applications to plant cell biology *in vivo*, we have explored a couple of strategies to cross cell wall and plasma membrane of plant cells that is generally impermeable for molecules exceeding 20-30 Da. Although it is possible to permeabilize the plasma membrane by mild detergents, this approach is not very useful for cell-biological applications, because the viability of the cells is dramatically affected, which is aggravated by the considerable turgor pressure (5-10 Bar) exerted by the expanding vacuole upon the inner face of the permeabilised membrane. We have established two physical approaches to address this issue:

(i) direct microinjection of large molecules into the cytoplasm using borosilicate needles [E1.5:14, E1.5:17].

(ii) application of high-voltage nanosecond pulses to protoplasts embedded in low-melting seaplaque agarose, followed by regeneration of the cell wall [E1.5:22].

However, these approaches require a high degree of manual skill and the yield of delivered particles is low. We therefore searched for approaches to permeabilize the plasma membrane. Although permeabilization with Triton-X 100 or dimethyl sulfoxide allowed to introduce nanocrystal-antibody conjugates into tobacco cells allowing for visualization of the various plant-microtubule arrays formed during the cell cycle [E1.5:10], this approach was not feasible for studies in living cells. The high-voltage nanosecond pulses had the drawback that they induced a bundling of actin filaments leading to changes in the flux of auxin [E1.5:1, E1.5:2, E1.5:6, E1.5:7, E1.5:9, E1.5:19, E1.5:21, E1.5:23], a central regulator of plant morphogenesis, and eventually apoptotic cell death [E1.5:22].

We decided therefore to test alternative approaches to introduce functional cargoes into plant cells.

Cell-permeating peptides: In cooperation with the group of Anne Ulrich we explored the potential of cell-permeating peptides. The critical point is the integrity of the vacuolar tonoplast membrane – if this membrane leaks, the vacuole will release large amounts of acidic and often toxic compounds that will kill the target cell within minutes. Exploiting the difference in charge between tonoplast and plasma membrane, it was possible to develop a permeabilization protocol using the CPP alamethicin [E1.5:24]. This protocol preserved viability of target cells that were even found to recover division after alamethicin had been removed. A simple and reliable quantitative assay to determine the pore sizes introduced by CPPs was developed. The exclusion size of the pores ranges between 4 and 10 kDa, which is still too small for nanoparticle-protein conjugates. Therefore, other CPPs such as harcianin B that were expected to produce larger pores, were tested. However, the size exclusion limits of the pores could not be increased sufficiently to allow passage of nanoparticles, and we therefore abandoned the approach to introduce membrane pores, but searched for CPPs that actually are able to entry by actual passage through membranes.

BP100, originally designed as an antimicrobial peptide against plant pathogens, was successfully employed as a fast and efficient cell penetrating agent to transport fluorescent test cargoes into the cytoplasm of walled plant cells. Uptake of BP100 proceeded slightly slower than the endocytosis of fluorescent dextrans used as endocytotic test tracer, but BP100 accumulated more efficiently and to much higher levels by an order of magnitude. The entry of BP100 was efficiently blocked by latrunculin B, suggesting that actin filaments were essential in the uptake mechanism [E1.5:26]. To test whether this novel transporter could also be used to deliver functional cargoes, we designed a fusion construct of BP100 with the actin-binding Lifeact peptide. We could demonstrate that the short BP100 (11 amino acids) transported the attached 17 residue sequence fast and efficiently into tobacco cells. The Lifeact construct retained its functionality and successfully labeled the actin bundles that tether the nucleus in the cell center (**Fig. 3**). By this proof-of-principle we demonstrated for the first time that actin can be visualized in living plant cells by chemical engineering without the need for genetic transformation [E1.5:26].

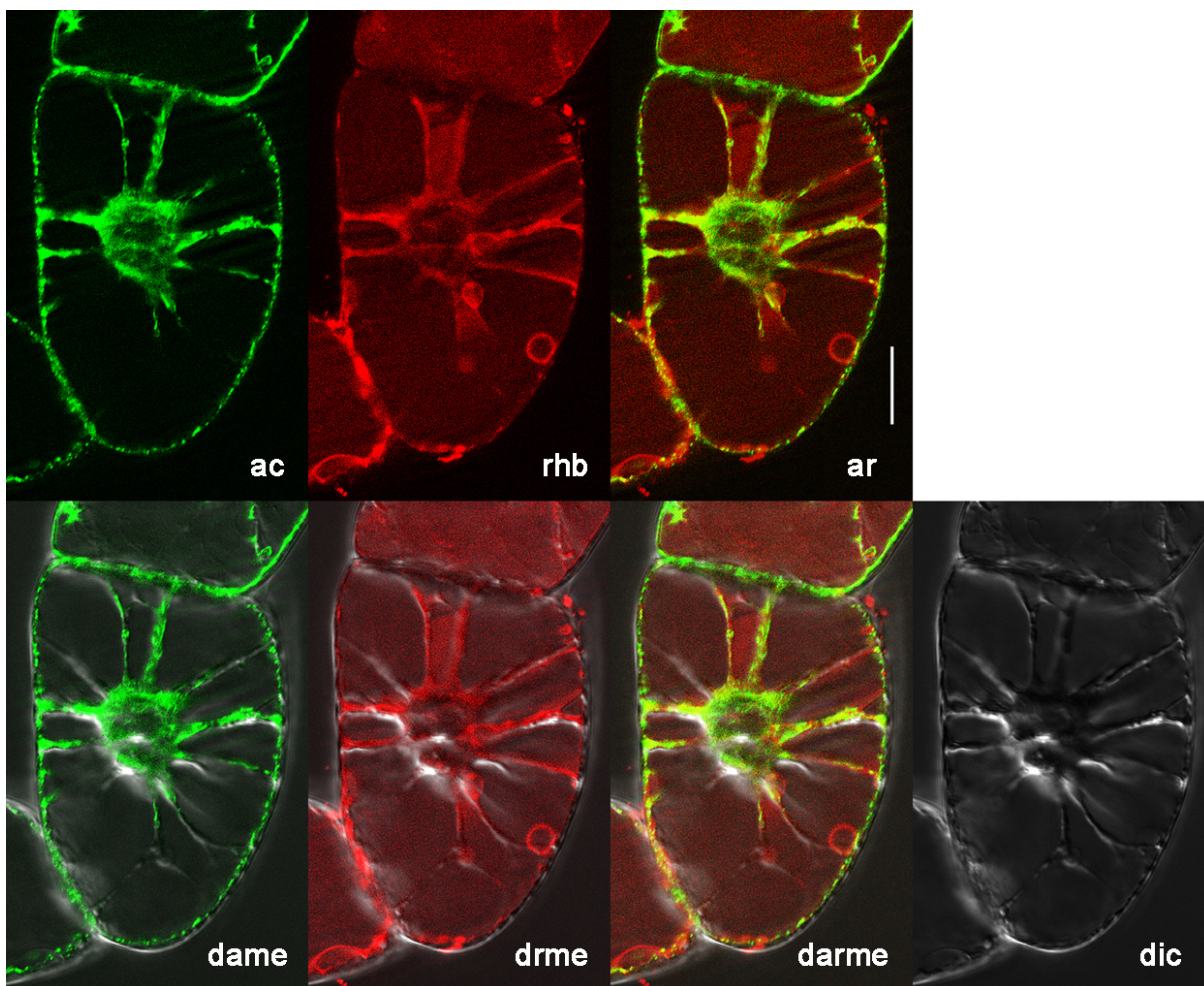


Fig.3: Localization of the RBL peptide (RhB-BP100-Lifeact) in individual cells of the line GF-11, where actin filaments are visualized by a GFP-tagged actin-binding protein. dic differential interference contrast; rhb signal for the RBL construct; drme merge of dic and rhb signals demonstrating that the RBL peptide enters the cells; ac signal for actin microfilaments; ar merge of the actin and the RBL signals producing a yellow signal from the strong colocalization of the peptide with the transvacuolar actin cables. Size bar 20 μm . Taken from [E1.5:26].

Trojan Peptoids: In parallel to the CPP approach, we made use of a cooperation with the group of Stefan Bräse that investigate Trojan Peptoids as carriers for the delivery of covalently attached cargo molecules of diverse chemical nature (oligonucleotides, proteins, fluorophores, synthetic DNA and even liposomes or nanoparticles) into target cells.

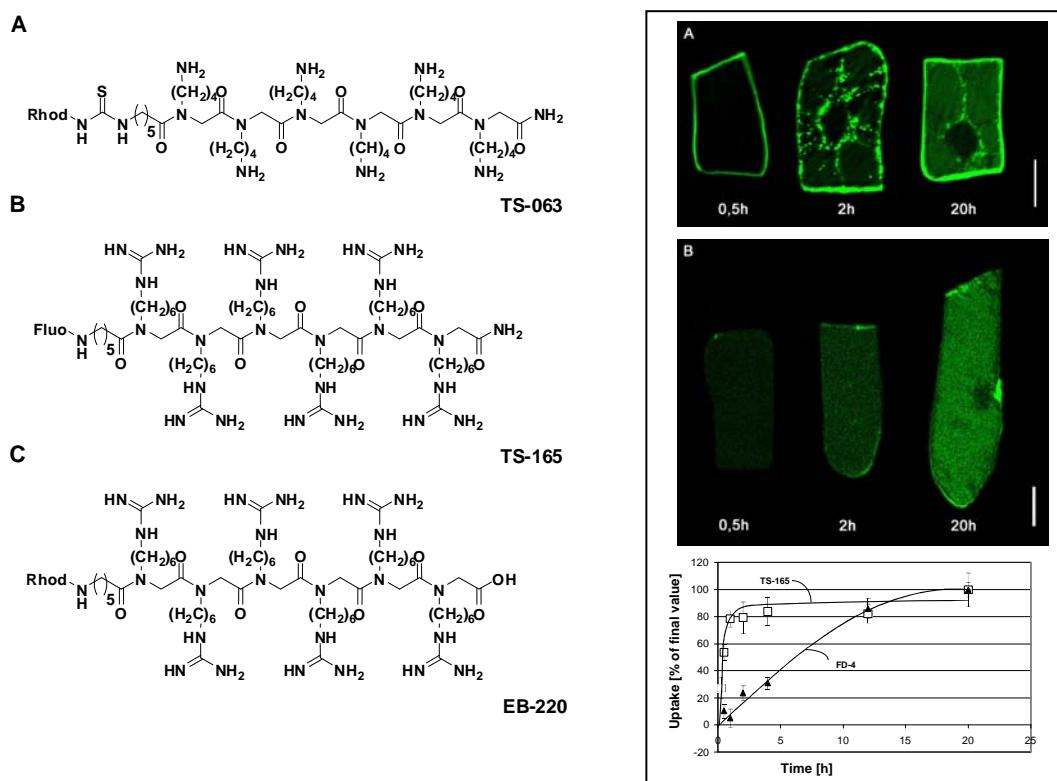


Fig.4: Structure of the Trojan Peptoids investigated (left). Uptake of the Trojan Peptoid TS-165 (right, A) in comparison to fluorescently labelled dextrane FD-4 (right, B) into living tobacco BY-2 cells. Size bar 20 μm . The time course of uptake was quantified by a microscopical assay based on quantitative image analysis (right, lower graph). Taken from E1.5:20.

Despite the potential of Trojan Peptoids as carriers, so far, this transport has only been demonstrated for mammalian cells and the actual cellular mechanism of membrane passage has not been elucidated. Plant cells, that are encased in a cellulosic cell wall and differ in membrane composition, represent an alternative experimental system to address this issue. We therefore investigated carrier-peptoids with or without guanidinium side-chains with regard to their uptake into plant cells, the cellular mechanism of uptake, and intracellular localization (**Fig. 3 left**).

We could show that, in contrast to polyamine peptoids (polylysine like), fluorescently labelled polyguanidine peptoids (polyarginine like) entered rapidly into tobacco BY-2 cells without affecting the viability of these cells. A quantitative comparison of this uptake with endocytosis of fluorescently labelled dextrans indicated that the main uptake of the guanidinium peptoids occurred between 30-60 min and clearly preceded endocytosis (**Fig. 3**). Dual visualization with the endosomal marker FM4-64 showed that the intracellular guanidinium peptoid was distinct from endocytotic vesicles. Moreover, Wortmannin, an inhibitor of receptor-mediated endocytosis, blocked the uptake of fluorescent dextrane as test cargo, but did not impair the uptake of Trojan Peptoids. Once the polyguanidine peptoids had entered the cell, they associated with actin filaments and microtubules (**Fig. 4**). By pharmacological manipulation of the cytoskeleton we tested, whether the association with the cytoskeleton was necessary for uptake, and observed that the actin inhibitor latrunculin B as well as the microtubule inhibitor oryzalin very efficiently impaired uptake and intracellular spread of the guanidinium carrier, which is consistent with rising evidence of a structurally tight interaction of the plant cytoskeleton with binding structures in the plasma-membrane [E1.5:15, E1.5:16].

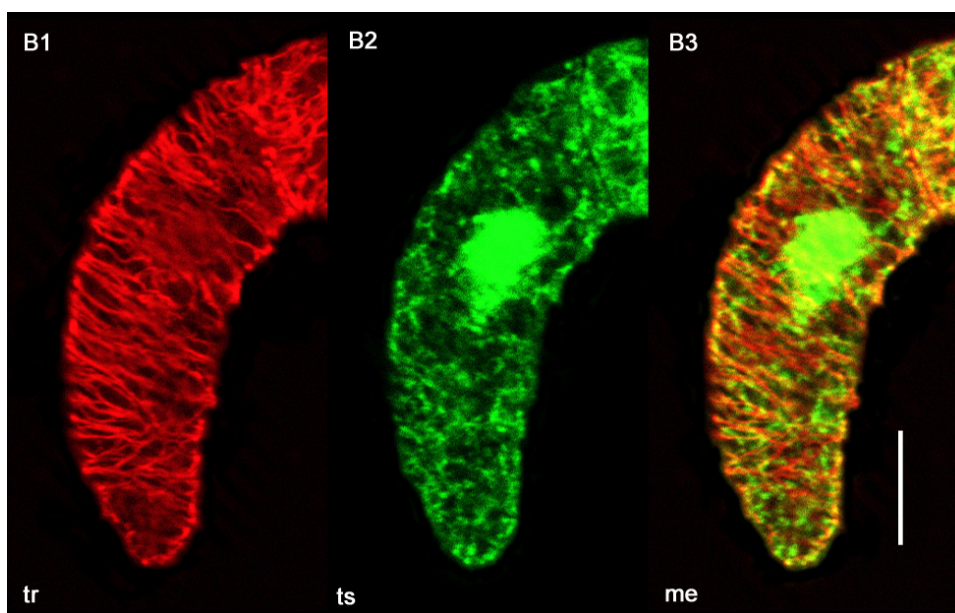


Fig.4: Dual visualization of the Trojan Peptoid TS-165 with microtubules. tr microtubule signal, ts signal for TS-165, me merge of both signals. Size bar 20 μm . Taken from E1.5:20

This approach for membrane passage into walled, intact plant cells was then extended by using Trojan Peptoids that can be targeted to specific intracellular targets. CPP mimetics based on a peptoid backbone containing guanidinium (arginine) side chains, which translocated readily through cell wall and plasma membrane, and accumulated rapidly in mitochondria of BY-2 tobacco cells. These peptoids were therefore named Mitochondria Penetrating Peptoids (MPPos). Covalent coupling to Rhodamine or the highly photostable fluorophore Atto655 allowed to follow the movement of individual mitochondria at high resolution in Stimulated Emission Depletion (STED) microscopy, and at high spatiotemporal resolution by Spinning-Disc Microscopy and Resonance Scanning 4D-Confocal Microscopy. We subsequently exploited the potential of MPPos for High-Speed 4D-CLSM to investigate the effect of actin polymerization and depolymerization on mitochondrial movement [E1.5:26]. Using pharmacological manipulation of the cytoskeleton and computational rendering of the high resolution images, we determined trajectories and velocities of individual mitochondria and could extract the contributions of actin polymerization and myosin activity to mitochondrial movement (**Fig. 5**).

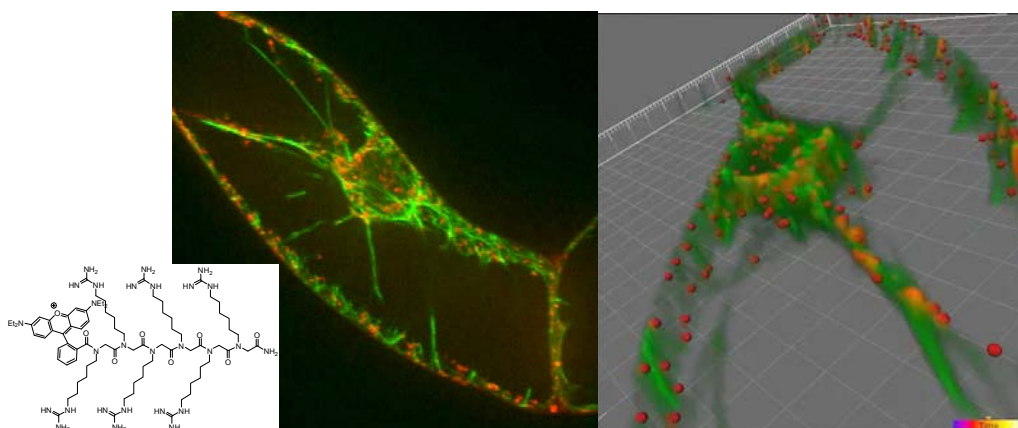
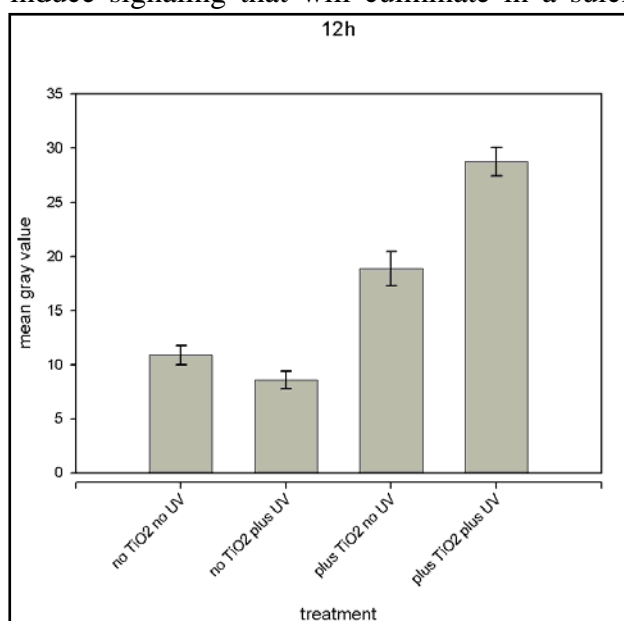


Fig.5: Dual labelling of tobacco cells for actin filaments (actin-binding domain of plant fimbrin in fusion with GFP), and mitochondria (red, using the Trojan Peptoid {RhodB}-**HEX**₆ NH₂), followed by Spinning-Disc Confocal Microscopy (left) and rendered for quantitative image analysis using the IMARIS-system. Taken from E1.5:20

3. Adaptation of nanoparticles for plant-cell biology

The motivation for this project has been the manipulation of plant cells by introducing nano-sized cargoes with specific properties. We have explored this along two lines: by adapting the nanosized cargo and by improving the specificity of the molecular transporter.

Adapting nanosized cargoes: After our demonstration that CdSe nanocrystals can be used for direct immunofluorescence of plant microtubules *in situ*, making use of the elevated fluorescence intensity of the CdSe nanocrystals [E1.5:10, E1.5:12, E1.5:24], it is now possible to circumvent visualize different monoclonal antibodies simultaneously without cross-reactions. However, for applications in living cells, the potential toxicity of Cd is vital. Making use of the possibility to synchronize the divisions of these cells, we followed the biological response to cadmium at different stages of the cell cycle. We could show that programmed cell death with apoptotic features such as internucleosomal DNA fragmentation was induced by cadmium in the S and G2 phases of the cell cycle, whereas in M and G1 phases the cells did not respond by apoptosis [E1.5:13]. From this study it can be concluded that even a low level of Cd-leakage that is not toxic *per se*, can induce signaling that will culminate in a suicidal response of the target cell, which is a further



argument in favour of silica shells over BSA ligands. We therefore have started to work with less toxic alternatives (ZrS, in cooperation with the group of C. Feldmann or InP, in cooperation with the group of T. Nann, Norwich). Recently, in cooperation with the group of L. Fruk, we have tested functionalized TiO₂ nanoparticles for their potential in plant cell biology. These particles can generate reactive oxygen species that trigger plant defence [E1.5:11] and culminate in a so called programmed cell death (the plant version of apoptosis) detectable by the TUNEL assay. Upon irradiation with a short UV-light pulse the generation of reactive oxygen species can be elevated. As expected, this increases the amplitude of the death response (Krahmer, Fruk, and Nick, unpublished, **Fig. 6**).

Fig. 6: occurrence of programmed cell death measured as normalized TUNEL signal in tobacco cells treated with bifunctionalized TiO₂ nanoparticles. The light-pulse used for activation of the particle activity does not cause any significant increase in cell death. However, the particles by themselves can stimulate programmed cell death, this increase is further elevated upon activation of the particles by a UV-pulse (Krahmer, Fruk, and Nick, manuscript in preparation.)

Improving the specificity of molecular transporters: In cooperation with the groups of S. Bräse and U. Schepers, we further developed the specificity of Trojan Peptoids with polyguanidine side chains. A third-generation derivative of the structures found to penetrate into tobacco cells [E1.5:12, E1.5:20] could be specifically introduced into plant mitochondria, such that the movement of mitochondria along actin filaments could be followed for the first time by high-resolution 4D confocal microscopy [E1.5:25] yielding high-quality information on individual trajectories that could be rendered and quantified in great detail using the IMARIS image analysis package leading to novel insights into the role of actin polymerization as driving force of mitochondrial movement. We are presently adapting these transporters for specific manipulation of plant mitochondria using light-activable porphyrin residues (in cooperation with the Balaban lab) in order to trigger

programmed cell death in specific cells of a file to investigate cell-cell communication. The CPP approach is extended by a panel of different peptide types that are presently screened for uptake kinetics and subcellular targeting in tobacco cells. Using actin nucleation as a proof-of-principle we want to expand our tool-kit for chemical engineering by introducing specific peptides that due to structural similarity with protein-protein interaction domains will compete with native proteins such that specific interaction of actin organization should become possible. A second interesting target are nuclear export signals on plant tubulin that might be used to engineer cells with elevated cold tolerance [1.5:4]

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