motion of the probe thus the target molecules of interest. Herein, we present an integrated imaging platform based on the combination of birefringent gold nanorod probes and differential interference contrast (DIC) microscopy that can achieve single particle orientation and rotation tracking (SPORT). This new technique allows us to resolve translational and rotational motions of gold nanorod probes at each stage throughout the clathrin-mediated endocytosis process, leading to the disclosure of their binding status on the cell membrane and characteristic rotational motions generated by protein modules.

2196-Pos Board B182
Particle Tracking of Membrane VAMP2 within Secretory Cells in the Nanometer Regime
Daniel Boening, Martin Wiemhoefer, Julia Trahe, Cora Sandra Thiel, Jurgen Klingauf.
In the past we suggested that a pre-sorted and pre-assembled pool of SV proteins on the presynaptic membrane might support a first wave of clathrin-mediated endocytosis. Recent evidence using IsoSTED nanoscopy of surface vesicular proteins indicates, that such pre-assembled patches exist at the periactive zone in hippocampal boutons.
Here we analyzed the diffusion kinetics of the surface-stranded vesicular protein Vamp2 coupled to the photoactivatable proteins Dendra and Eos by 3D particle tracking in combination with photo activation localization microscopy (PALM). The z-position information of the fluorophores was estimated by measuring the widths of the elliptical PSF caused by a cylindrical lens in the detection pathway. The elliptical PSF is translated into axial positions by a least squares fit of the ellipticity. The axial position estimation can then be enhanced by Gaussian image filtering with a resolution enhancement of at least 30%.
With 3D particle tracking in living secretory cells like PC12 or hippocampal boutons nanostructures of faster and slower diffusion or transport of a VAMP2-EOS complex were identified. From 3D particle tracking maps we can show highly heterogeneous diffusion behaviour of Vamp2 within PC12 cells and synapses. This enables us to resolve and characterize areas of endocytosis within living cells.

2197-Pos Board B183
Superresolution Analysis of syb2 Distribution in Membranes of Live and Fixed Cells
Martin Wiemhoefer, Julia Trahe, Cora Thiel, Jürgen Klingauf.
Imaging of continous biological structures is diffraction limited in far field microscopy due to the overlap of the point spread functions (PSFs) of the single fluorescent emitters. Superresolution beyond the diffraction limit by photoactivation localization microscopy (PALM) has therefore become indispensible for studying biological processes.
By combining PALM with Total Internal Reflection Microscopy (TIRF) we were able to localize single molecules involved in secretion in fixed and live neurosecretory cells. The fusion of secretory vesicles with the membrane is mediated by the assembly of low-energy complexes formed by the coil-coiling of three members of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein family: SNAP-25, syntaxin, and synaptobrevin 2 (syb2).
Now a dimerization of the syb2 via transmembrane domains (TMDs) in intact PC12 cells has been reported [1]. However it is not clear if such interactions have an effect on the kinetic properties of fast vesicle fusion. To this end we expressed syb2 fused to Dendra2 in HeLa and PC12 cells and mapped the protein distribution in membranes of live and fixed cells. We found evidence for a dimerization of syb2/Dendra2 by comparison with cotransfection of untagged syb2 in HeLa cells and quantified the protein distribution in the cellular membranes.
TMD mutants display slight inhibitory and enhancing effects on membrane fusion as described in the literature [1]. To further characterize the TMD-mediated interactions between syb2 molecules in intact cells we studied the distribution of TMD mutants in live and fixed cells and quantified density and distance to study the role for the syb2 TMD in exocytosis.
[1] Fdez et al, 2010.

2198-Pos Board B184
Contributions of CryoeM to Visualize Membrane-Curvature Generation - Mechanisms and Implications
Vinzenz M. Unger, Carsten Mim.
Protein mediated recognition, maintenance and modulation of membrane curvature plays critical roles in many cellular processes. Based on a growing number of crystal structures from the BAR-superfamily of proteins, MD simulations and spectroscopic work, it now is widely accepted that scaffolding and/or insertion of amphiphatic wedges play important roles in the curvature related processes. What had been missing from the picture was a direct visualization of BAR-domain proteins in their membrane bound states. Addressing this need through use of electron cryomicroscopy, our previous work on F-BAR domains confirmed that these modules induce curvature by scaffolding, but also showed that these domains can engage flat bilayers through an alternate binding interface that is physiologically relevant. Extending our work to the N-BAR family, it now becomes apparent that these modules function very differently from their F-BAR cousins, and that the specific design principles of N-BAR lattices have direct implications for the interaction of these scaffolding components with downstream interaction partners.

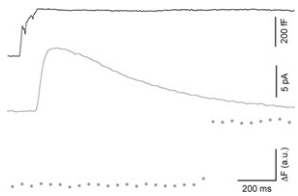
2199-Pos Board B185
Membrane Deformation Caused by Clathrin and Associated Adaptor Proteins In Vitro
Mohammed Saleem, François-Damien Delapierre, Laurent Malaquin, Aurelien Roux.
Clathrin-dependent endocytosis is a common mode of cellular trafficking. The formation of clathrin-coated vesicles is preceded by deformation of membrane due to the interacting coat proteins comprising of clathrin and various adaptors. However, the precise mechanism behind the deformation of membrane and existence of diverse shapes, sizes and symmetry remains unknown. We intend to develop a bio-mimetic system to allow the study of the precise biochemical and physical parameters that control the rate of clathrin polymerization and to determine the membrane deforming forces. Clathrin binding to giant unilamellar vesicles was followed using fluorescent clathrin and the coat polymerization was confirmed by fluorescent recovery after photobleaching (FRAP). Interestingly, clathrin binding and polymerization on membrane takes place only when AP180 is present in the injected mixture of proteins. We also observed significant differences in the membrane deformation caused by various adaptor proteins (AP180 and AP 1, 2) and clathrin by differential interference contrast fast video microscopy (DICM), on a micron scale. Additionally, interesting insights on the topology of the membrane deformations caused by various adaptor/clathrin mixtures on membrane sheet assemblies were obtained by means of atomic force microscopy (AFM). Our findings suggest that AP180/clathrin lead to the generation of extensive bud like structures that are ~100nm in diameter and ~20nm in height. Further, high resolution imaging will be used to differentiate various polygonal transformations in clathrin lattices under varying conditions of adaptor/clathrin, which may define various shapes and sizes of buds. In addition, precise forces and actors generating the forces behind membrane deformation and the role of pre-induced membrane curvature in the clathrin polymerization and varying lattice structure will be investigated.

2200-Pos Board B186
Quantitative Analysis of Membrane Deformation and Fission Induced by Dynamin GTPase Activity
Sandrine Morlot, Martin Lenz, Jacques Prost, Jean-François Joanny, Aurélien Roux.
Dynamin is widely used by cells to sever lipid bilayers. During this process a short helical dynamin polymer (1 to 3 helical turns) assembles around a membrane tubule and reduces its radius and pitch upon GTP hydrolysis. This deformation is thought to be crucial for dynamin's severing action and results in an observable twisting of the helix [1]. Here we quantitatively study the factors determining the dynamics of this deformation by studying long dynamin. We perform in vitro experiments where we attach small beads to the dynamin helix and track their rotation in real time, thus collecting information about the space and time dependence of the deformation. Longer helices deform more slowly as predicted by a generalized hydrodynamics theoretical model [2]. Further agreement between experiments and theory indicates that the concerted deformation dynamics is dominated by the draining of the membrane out of the helix, allowing us to quantitatively characterize helix-membrane interactions [3]. We also study the dynamics of tube fission induced by dynamin GTPase activity. Membrane nanotubes are pulled from Giant Unilamellar Vesicles (GUV) using optical tweezers and membrane tension is set by aspirating the GUVs within a micropipette. Dynamin and GTP are injected near the tube. Tubes always break few seconds after dynamin starts polymerizing around the tube. We show that probability of fission depends on GTP concentration, no global depolymerization occurs during GTP hydrolysis and membrane geometry affects fission.

[1] Aurélien Roux, Katherine Uyhazi, Adam Frost, and Pietro De Camilli. Nature 2006.
[2] Martin Lenz, Jacques Prost, and Jean-Francois Joanny. Phys. Rev. E 2008.
[3] Sandrine Morlot, Martin Lenz, Jacques Prost, Jean-Francois Joanny, Aurélien Roux. submitted

2201-Pos Board B187
GED-Zippering Induced by PH Domain-Membrane Insertion Elicits Dynamin Assembly-Dependent GTPase Activation
Rajesh Ramachandran.
The multi-domain GTPase dynamin catalyzes the scission of deeply invaginated clathrin-coated pits (CCPs) from the plasma membrane employing domain-specific protein-membrane and protein-protein interactions. By monitoring the dynamics of pleckstrin homology (PH) domain-mediated dynamin-membrane, and GTPase effector domain (GED)-mediated dynamin-dynamin interactions in real time, the requirement for allosteric conformational coupling in dynamin GTPase activation is revealed. Membrane insertion-defective dynamin-1 mutant, Dyn1 I533A uncouples intermolecular GED-GED interactions from PH domain-membrane interactions, and is therefore rendered incompetent in eliciting assembly-dependent GTPase activation. Therefore, stable PH domain-membrane interactions allosterically trigger intermolecular GED-zippering to effect dynamin assembly-dependent GTPase activation during clathrin-mediated endocytosis.

2202-Pos Board B188
Serotonin Release and Matrix Decondensation Precede Dissipation of the Proton Gradient in Secretory Granule Exocytosis
Daniel Steinbrenner, Jan C. Behrends.
Exocytosis is a rapid but complex reaction involving multiple steps from the initial protein interactions to formation of the fusion pore and release of vesicle content. The latter is generally viewed as the last step in the sequence. For secretory granules of mast cells, it has been proposed that dissipation of the granular proton gradient is an early event, permitting decondensation (swelling) of the granule matrix and subsequent release of small molecule cargo, such as serotonin (Willams and Webb 2000, J.Cell Sci. 113, 3839). Here, we have examined this issue using simultaneous capacitance recording and amperometry as well as optical (fluorescence and transmitted light) recording. Using RBL-1 and RBL-2H3 cells loaded with serotonin and transfected with synaptpHluorin as a reporter of intragranular pH, we find that, in contrast alkalinization of the granules is the last event in the series, lagging by several 100 ms both serotonin release and matrix decondensation, which start immediately after fusion pore expansion. Our finding is in line with the idea that granular pH change is greatly delayed by the strong buffering capacity of both serotonin and the proteoglycan matrix.



2203-Pos Board B189
The Conformational Change of SNAP25 during the Exocytosis
Ying Zhao, Qinghua Fang, Khajak Berberian, Kassandra Kisler, Wolfhard Almers, Manfred Lindau.
It is widely assumed that a conformational change in the SNARE complex induces fusion pore opening and transmitter release. The SNAP-25 FRET construct SCORE (SNARE complex reporter) has CFP as FRET donor inserted at the N-terminal end of SN1 and the fluorescent protein Venus (YFP) as FRET acceptor inserted at the N-terminus of SN2. It was previously shown that SCORE that reports a FRET change in response to stimulation that is calcium dependent but is not abolished by tetanus toxin treatment, which abolishes fusion (An and Almers 2004 Science 306:1042). To determine if the conformational change reported by SCORE is directly associated with fusion we monitored SCORE fluorescence in chromaffin cells in TIRF mode while exocytotic events at the cell bottom were spatially and temporally monitored by a microfabricated electrochemical detector (ECD) array with four Pt electrodes patterned on a glass coverslip (Hafez et al. PNAS 2005 102:13879). After the SCORE-expressing cells were placed on the top of the ECD array, the amperometric currents and fluorescence images were simultaneously recorded. Based on the charge ratios of oxidation currents recorded by the four electrodes, the localization of single exocytotic events was determined. The time course of CFP and YFP fluorescence in the 4 camera pixels at the release site was extracted for 10 s time intervals before and after the amperometric spike. Averaging >300 events revealed a transient increase of YFP and a transient decrease of CFP fluorescence by ~2.5% at the time of amperometric events, The increased FRET level returned to the pre-exocytotic level within ~5s. This result directly indicates that the SNAP25 conformational change is directly associated with fusion. Supported by NIH grant R01GM085808.

2204-Pos Board B190
On-Chip Electroporation Results in Calcium- and Chloride- Stimulated Exocytosis Assayed with Planar Electrochemical Microelectrodes
Jaya Ghosh, Xin Liu, Kevin D. Gillis.
We are developing microfabricated devices consisting of arrays of electrochemical electrodes in order to increase the throughput of single-cell measurements of quantal exocytosis from neuroendocrine cells and to develop technology that allows simultaneous electrochemical detection and fluorescence imaging of single fusion events. One component of this effort is to develop on-chip methods for stimulating exocytosis from select cell population on the chip. We have demonstrated that the same electrochemical microelectrode can be used to electroporate an adjacent cell and then measure quantal exocytosis using amperometry. Trains of voltage pulses, 5-7 V in amplitude and 0.1-0.2 ms in duration, can reliably trigger exocytosis in Ca^{2+}-containing bath solutions using gold and diamond-like-carbon electrodes. Electrodes fabricated from Indium-Tin-Oxide were less effective at eliciting exocytosis via electroporation. Higher rates of exocytosis were observed with bath solution containing higher $[Ca^{2+}]_e$, and experiments with semi-transparent electrodes and the Ca^{2+} indicator fura-4F demonstrate a rise in $[Ca^{2+}]_i$ upon electrical stimulation. Little or no exocytosis was observed in Ca^{2+}-free (5 mM EGTA) solutions with glutamatic acid as the major anion. Surprisingly, exocytosis could be elicited upon electroporation in 0 Ca / 5 mM EGTA solutions if the bath contained high Cl^- concentrations (140 mM NaCl or KCl). This is surprising since the same solution does not induce exocytosis when loaded into the cell through a patch pipette during whole-cell recording. We conclude that electric fields sensitize granules to undergo exocytosis in response to Cl^-. Supported by NIH R01 NS048826.

2205-Pos Board B191
Functional Characterization of Putative Synaptotagmin-Binding Interfaces in SNAP-25
Ralf Mohrmann, Heidi De Wit, Emma Connell, Bazbek Davletov, Matthijs Verhage, **Jakob B. Sørensen**.
SNARE complexes possess multiple negative surface charges that probably mediate association with regulatory factors during exocytosis. Several distinct groups of negatively charged residues in SNAP-25 have been identified as essential for binding synaptotagmin-1 (syt-1). One group exists at the C-terminal end (D172/D179/D186/D193), while another site extends around Layer 0 (D51/E52/E55). A third potential group may consist of D166/E170, as we previously found that E170 is essential for fast release. To investigate the nature of the binding interface, we introduced alanine-substitutions at the three putative binding sites and characterized the resulting mutant variants after expression in SNAP-25 -/- chromaffin cells. Secretion was induced by photolysis of caged-Ca2+ and assayed by capacitance measurements and amperometric recordings. We found substantial differences in the phenotypes of these mutant variants, which excludes that all sites cooperate in the same mode of syt-1 interaction. Though no variant fully phenocopied the changes seen in syt-1 -/- cells, SNAP-25A D51A/E52A/E55A caused a slowdown of secretion reminiscent of the syt-1 -/- phenotype. SNAP-25A D166A/E170A decreased total release substantially and abolished the fast burst component, while SNAP-25A D172A/D179A/D186A/D193A had little effect on secretion. Pull-down assays demonstrated that mutation of the two groups around Layer 0 indeed restricted syt-1 binding, while mutation of the C-terminal site did not decrease affinity towards syt-1. By electron microscopy we found that all mutations significantly affected docking of vesicles, which depends on both SNAP-25 and syt-1 (De Wit et al., 2009, Cell 138:935-946). This implies that SNAP-25/Syt-1 assisted docking might rely on another mode of interaction between SNAP-25 and syt-1 than fusion triggering. Our data thus suggest that the interaction between SNAREs and syt-1 might involve multiple interaction modes, and that negative charges around Layer 0 most likely function as a syt-1 binding interface during exocytosis triggering.