In mouse chromaffin cells expressing green synaptopHluorin in the granules, cell membranes were stained from the outside with red lipophilic dyes that rapidly redistribute by flip-flop between both leaflets. Fusion of secretory granules was monitored by evanescent wave microscopy. Exocytosis was triggered by superfusion with high K+ solution, and double images were taken at 491 and 560 nm excitation, respectively. Fluorescence signals of the membrane probes recorded in the red channel were spatially and temporally aligned with respect to fusion events in the green channel to yield average movies with high signal-to-noise ratio. We found the membrane fluorescent signals to be slightly increased in diffraction-limited spots at locations of docked granules for up to a second prior to fusion. The fluorescent signals, however, rapidly decreased to background levels upon fusion of the granules at the respective sites, with the fluorescence dissipating from the center to the periphery.
Our results are best explained by mixing of lipids prior to fusion in a hemifused state.

3540-Pos
Towards Real Time Analysis in Photoactivation Localization Microscopy
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We investigated a new fast and precise algorithm for analysis of Photoactivation Localization Microscopy (PALM) images, based on real-time template matching used e.g. in face recognition or astrophysics. For generation of the template we measured the Point Spread Function (PSF) with high precision for the experimental setup in use and modelled it by a 2D Gaussian. This Gaussian is now used as template, and the problem of image registration is solved by a fast Sum Square Difference (SSD) Algorithm [1].
To gain subpixel resolution we used a newer class of algorithms [2]. By resampling the template and using an error correction method a maximum error of 10% of the pixel period is expected. The performance is tested in comparison to established algorithms in PALM with regard to computation time and accuracy.
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). However it is controversially debated how many SNARE complexes are minimally needed for SV priming and fusion. To this end we expressed syb2 fused to Dronpa or Dendra2 in PC12 cells. With the help of new algorithms real-time localization and counting of syb2 with sampling rates up to 10Hz was possible.
Thus, template matching is a promising strategy towards real-time analysis in FPALM, whenever direct evaluation of recorded images is needed for proper object selection like in live cell imaging.
[1] Barnea u. Silverman, 1972.
[2] Frischholz u. Spinnler, 1993.

3541-Pos
Syntaxin1a Dispersion and Assessment of *cis*-Snare-Complex Formation-Disassembly during Synaptic Transmission in Hippocampal Neurons
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Soluble-N-ethylmaleimide-sensitive-factor-attachment-protein-receptor(SNARE)-complex formation and dissociation are biochemical prerequisites of synaptic transmission that occur during calcium-triggered synaptic vesicle (SV) fusion and subsequent recycling, which prepares cellular machinery for the following responses to action potentials (AP). The exact sequence of molecular events remains unknown, and it's a technological challenge to demonstrate SNARE-protein interactions *in-situ*, during AP-induced synaptic transmission. In this study, C-termini-labeled fluorescent constructs of Syntaxin1a-Cerulean and VAMP2-Citrine were transiently expressed either alone or together in cultured hippocampal neurons. A Syn1a-Cer-probe revealed a more uniform distribution of fluorescence in the plasma-membrane, with slightly higher intensity in synaptic boutons, while VAMP2-Cit-fluorescence was predominantly in boutons, suggesting that trafficking of heterologously-expressed constructs is similar to endogenous SNAREs. Efficiently transmitting boutons were identified by FM4-64-destaining during electrical-stimulation. These boutons revealed both faster transient increase of VAMP2-Cit-fluorescence (synaptopHluorine-effect), and a slower decrease of Syn1a-Cer-fluorescence. The latter effect started with a 3-6 s delay after beginning 30 Hz-stimulation, and developed as stimulation continued for 10-30 s. The largest reduction for averaged data was seen 5-10 s after stimulation completed, and recovery occurred in the following 20-40s. The decrease of Cer-fluorescence that occurred in regions of intensive FM-destaining was often accompanied by a transient increase of fluorescence in adjacent areas. In transmitting boutons co-expressing both Syn1a-Cer and VAMP2-Cit probes, the reduction was slightly stronger (up to 3-4%, although not significant yet, $P<0.15$) compared to the Syn1a-Cer alone (1-2%), even though the latter boutons showed on average larger FM-destaining. The decrease remains if measured in larger areas that presumably include dispersed probe. We hypothesize that a fraction of the Cer-fluorescence reduction can be attributed to FRET between Syn1a-Cer probe as a fluorescence-donor and VAMP2-Cit as an acceptor, which come in close proximity upon cis-SNARE-complex formation after SV-fusion.

3542-Pos
Synaptotagmin-1 and Complexin-2 Work Cooperatively to Promote Exocytosis from Adrenal Chromaffin Cells
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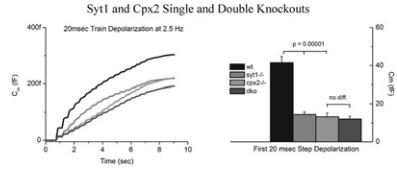
Neurons create secretory vesicles and sites for exocytosis that together promote rapid formation of exocytotic pores in response to fluctuations in $[Ca^{2+}]_i$. The SNARE complex is perfectly positioned to influence this process, and it provides points of entry for accessory SNARE proteins Synaptotagmin1 and Complexins. Recent biochemical studies have proposed that a Syt1-Cpx interaction facilitates membrane fusion, yet earlier studies proposed a competitive relationship. No physiological study has directly addressed this, so here we measured release from chromaffin cells derived from Syt1 and Cpx2 single and double knockout animals. Trains of step depolarizations varying in length were given to probe the number of vesicles in the rapid and sustained release phases. The change in δC_m after 20 and 100msec steps was reduced by ~70% with loss of a single gene, but deletion of Syt1 on the cpx2-/- background (dko) was without consequence (see Figure). Syt1-/- cells showed the greatest recovery in release with more time and Ca^{2+}. Finally, amperometric spike properties were altered. In total, Syt1 and Cpx cooperate in a positive fashion to increase the probability of release and the size of single events.



3543-Pos
Complexin and Synaptotagmin in Synaptic Function and Plasticity
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Neurotransmitter-release is a specialized form of vesicle fusion that shares a common SNARE-mediated fusion mechanism with other vesicle trafficking pathways within cells. With the emergence of multicellular organisms and just before of the formation of the primitive nervous system, two principal families of SNARE complex-binding proteins emerged, synaptotagmins (Syt) and complexins (Cpx). Current data suggest that SNARE binding by the two proteins allows Syt-1 to promote fusion in a calcium dependent manner, while Cpx prevents premature fusion in the absence of calcium influx. However, their precise roles in regulated secretion and effects on short-term synaptic plasticity (STP) is poorly understood. Here, we present a thorough analysis of synaptic transmission at NMJs of mutant and overexpression animals using recordings of evoked and spontaneous EPSCs using voltage-clamp at the *Drosophila* NMJ. Kinetic analysis of eEPSCs reveals that synchronous and asynchronous release critically depends on the level of Cpx and Syt expression. Moreover, sEPSCs analysis indicates that spontaneous release is tightly regulated by Syt and Cpx. Additionally, Syt and Cpx protein levels alter quantal content and STP, suggesting that these proteins play a role in the availability of vesicles for fusion. We propose that Syt minimizes the energy of the transition-state for SNARE-complex fusion, while Cpx decreases this free energy, creating an energetic barrier at a late step of vesicle fusion clamping vesicles in the immediately releasable pool. Finally, we tested the functional conservation of Cpx comparing the two *Drosophila* splice variants and the mammalian isoforms confirming their roles as a vesicle fusion clamp, but also revealing functional diversity in evoked responses.