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## Pair correlation analysis of Fixed PALM and Powerspectral Point Analysis of Live PALM applied on AQP3

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Water transport across the plasma membrane is mediated by aquaporin (AQP) water channels. In the kidney, water is reabsorbed from the renal filtrate through apically localized AQP2 and exits the cells through AQP3 and AQP4, which are expressed in the basolateral plasma membrane, where AQP3 is the main exit pathway.

The hormone Arginine Vasopressin (AVP) increases urine concentration via an increase in cAMP, leading to insertion of AQP2 containing vesicles into the apical plasma membrane. We previously found that a short-term increase in cAMP leads to an increase in lateral diffusion of AQP3, revealing short-term regulation. To further study if AQP3 is regulated at the nanoscale level, we first combined single molecule detection using Photoactivatable Localization Microscopy (PALM) imaging of fixed cells with pair correlation (PC). This showed that AQP3 organize in nano-domains smaller than 60 nm and upon stimulation mimicking vasopressin, changed organization to 60 – 200 nm sized nano-domains. Thus, PC-PALM revealed regulation at the nanometer resolution.

Furthermore, we performed live-PALM of AQP3 upon cAMP stimulation and have done analysis by power spectral analysis. This is the first time live-PALM data is analyzed by power spectral analysis. The analysis was done by first identifying isolated spots and fitting with a two-dimensional point-spread function. The localization errors were found theoretically and the diffusion coefficient for each trajectory was calculated using a covariance-based estimator. To demonstrate that the considered molecules were indeed freely diffusing with identical diffusion coefficients, we calculated the power-spectrum of each trajectory. The power-spectral values were rescaled with their expectation values given theoretically as a function of the averaged diffusion coefficient and the localization errors. The power spectral analysis did not show increased diffusion coefficient upon cAMP stimulation compared to the controls.

Thus fixed PALM revealed that AQP3 changed nanoorganization in the plasma membrane upon stimulation mimicking vasopressin; from an even distribution to an organization in nanoclusters. This indicates short-term hormone regulation of AQP3 at the nanoscale level, which may be important in urine concentration. PC-PALM may be used to reveal so far undetectable protein regulation at the nanoscale. We furthermore did live-PALM and showed an increase in diffusion after cAMP stimulation using power spectral analysis.