University of Southern Denmark

Quantum Dot Conjugations and Single Molecule Multicolor Imaging of Plasma Membrane Proteins and Lipids In Living Cells

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Abstract

Quantum dots (Qdots) are fluorescent nano-particles with a very high signal intensity and signal stability as compared to conventional fluorescent probes. Qdots are tunable in emission wavelength while all being excitable at the same excitation wavelength. Qdots are available with both primary amines and carboxyl reactive groups on the surface and thereby allows for conjugation of essentially any biomolecule of interest.

We have designed, made, and validated Qdots with different biomolecule substrates conjugated to the surfaces for specifically targeting native and artificial tags on plasma membrane proteins and lipids. By using Qdots of different colors imaging can easily be done with simultaneous visualization of up to four different molecular species at single molecule sensitivity and millisecond time integration. We find that this technique is very useful for studying the spatial and temporal nano-organization in the cellular plasma membrane of live mammalian cells.

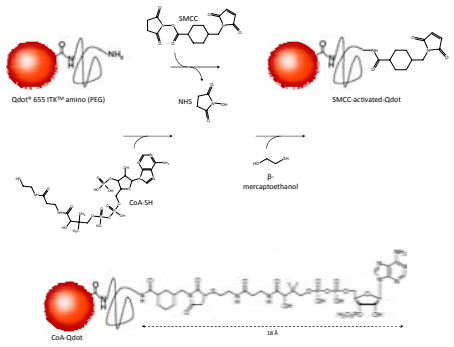
Summary

Our main interest is in using Qdots in single molecule imaging microscopy for investigating the spatial and temporal nano-organization of the cellular plasma membrane. As part of this work, we are interested in investigating various methods for targeting Qdots with high specificity and avidity to a variety of plasma membrane proteins and lipids. We are also interested in the single molecule behavior of Qdots (i.e. brightness, photostability, and spectral separation) this is however not included on this poster.

Qdot Conjugation Systems

Biomolecules can be conjugated to Qdots via either direct reaction with the chemical group on the Qdot surface or via reaction with a bifunctional cross-linker molecule reacting with the Qdots.

Qdots with Co enzyme A (CoA) moieties conjugated to the surfaces were made using the heterobifunctional cross-linker SMCC.

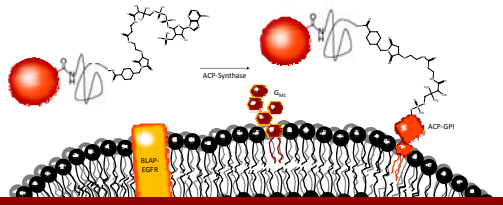


Targeting Strategy

The biomolecules target either native tags on cell membrane proteins and lipids or artificial tags added to a plasma membrane protein of interest and expressed via transfection of a genetic modified plasmid.

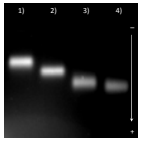
CoA targets specifically the 77 amino acid acyl carrier protein (ACP)-tag. This tag is artificially expressed as a glycosylphosphatidylinositol (GPI)-anchored fusion protein in the outer leaflet of the plasma membranes.

In presence of the enzyme ACP Synthase CoA-Qdots are covalently attached to the ACP-tagged GPI-anchor (ACP-GPI).



Validation of CoA-Qdot Conjugation

To validate the conjugation of CoA to the Qdots, the supposed CoA-Qdots were loaded on an agarose gel to visualize differences in surface charge as a result of the conjugation. Different molar ratios of CoA and Qdots were evaluated. The differences in migration of the Qdots at different stages in the conjugation confirms that CoA-conjugation is successful.



The separation between the bands in lane 1) and 2) shows that all Qdots are activated with the cross-linker and the separation between the bands in lane 2) and 3) shows that all activated Qdots are conjugating with CoA.

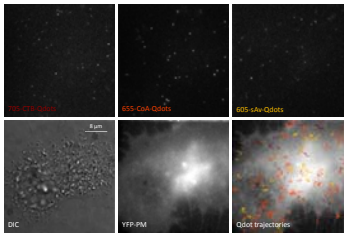
The overlap between the bands in lane 3) and 4) shows number of CoA per Qdot is not well-defined.

2 % agarose gel of CoA-Qdots at different stages of the preparation.
1) Un-conjugated Qdots, 2) Cross-linker activated Qdots,
3) CoA-Qdots (10:1), 4) CoA Qdots (20:1)

Multicolor Single Molecule Imaging of Proteins and Lipids

Our goal is now to implement and apply the 655-CoA-Qdots targeting the ACP-GPI together with other targeting systems in order to investigate the nano-organization of the cellular plasma membrane by high-speed, multicolor, simultaneous single molecule imaging of a combination of membrane proteins and lipids. The other targeting systems that we use are 605-streptavidin, 605-sAv-Qdots, targeting a biotinylated biotin ligase acceptor peptide (BLAP) version of the receptor tyrosine kinase epidermal growth factor receptor, BLAP-EGFR, and 705 cholera toxin B, 705-CTB-Qdots, targeting the ganglioside G_{M1}.

A preliminary example of such a combination of Qdots of three different colors targeting three different plasma membrane species are shown below. The cell under investigation furthermore expresses the yellow fluorescent protein (YFP) fused to the plasma membrane marker K-Ras2, YFP-PM, so the plasma membrane is visualized. Fluorescence images were acquired simultaneously using a Hg arc lamp, a 470/40 nm band pass excitation filter, a 100x, 1.3 NA objective, a 510LP emission filter with 5 ms integration times using a QuadView emission splitter and an Andor EMCCD camera at 30Hz and in the presence of 200 µM DTT to prevent Qdot color shifting and minimize Qdot blinking (two artifacts of Qdots).

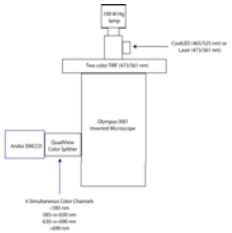


Snap-shots from the three different Qdot recording channels.
Left: 705-CTB-Qdots which target ganglioside G_{M1}.
Center: 655-CoA-Qdots which target GPI-anchored ACP.
Right: 605-sAv-Qdots which target biotinylated receptor tyrosine kinase BLAP-EGFR.

Cell images.
Left: DIC image of the cell under investigation.
Center: Sum of all frames in the YFP recording channel.
Right: Overlay of recorded trajectories of the three different kinds of Qdots on the YFP image.

Microscopy Set Up

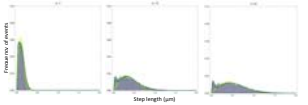
Our microscope is an inverted Olympus IX-81 microscope equipped for fluorescence excitation with a 100 W Hg arc lamp, a CoolLED (465 nm and 525 nm) and a laser (473 nm). Imaging can be done in both epi-fluorescence and TIRF mode. On the detection side we have a QuadView image splitter for simultaneous image acquisition in up to four separate emission channels and a very sensitive Andor EMCCD camera.



Tracking Analysis

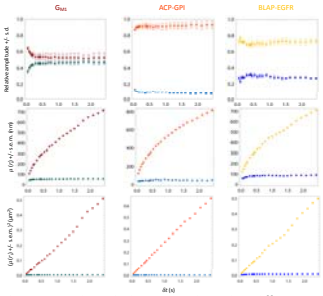
The position of each Qdot is linked between each frame to get single molecule trajectories from which we calculate the step-size, r, distributions $q(r,\delta t)$ at different time-intervals, δt. The step-size distributions are then fitted to the theoretical distribution for normal diffusion of one and two components of relative amplitudes a_1 and a_2 and diffusion coefficients D_1 and D_2 given by

$$q(r,\delta t) = a_1 \frac{r}{2D_1\delta t}\exp\left(\frac{-r^2}{4D_1\delta t}\right) + a_2 \frac{r}{2D_2\delta t}\exp\left(\frac{-r^2}{4D_2\delta t}\right)$$



Step-size distributions at different time-intervals.
The step-size histograms are fitted to one, light green, and two, dark green, components at different time-intervals (shown here δt = 1, 13, 25)

From the step-size distribution fits of two different mobile components the relative amplitude of the fast and the slow components, the mean step-size, and the mean-square step-size is obtained as functions δt



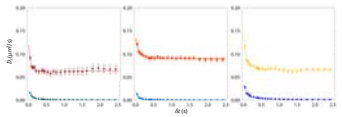
Relative amplitudes of the fast and slow component from the two component fits.

Mean step-size of the fast and slow component from the two component fits.

Mean-square step-size of the fast and slow component from the two component fits.

These are used to calculate a time-dependent diffusion coefficient, which is fitted to a single exponential decay, by

$$D_1(t) = D(t \to \infty) + (D(t \to 0) - D(t \to \infty))\exp(-k_D t)$$

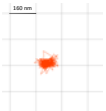


Time-dependent diffusion coefficient of the the fast and slow component from the two component fits.

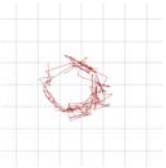
We find confined areas of the investigated molecules with sizes in the order of 30 -100 nm, and the next step is to treat the plasma membrane in various ways to find the molecular reason of the confinement.

Also, we need to implement analysis at the single trajectory level to give a better description of the observed trajectories.

Example of a more freely moving BLAP-EGFR molecule, which shortly shows confinement .



Example of confined motion of a ACP-GPI molecule.



Example of a very interesting behavior of a circling G_{M1} molecule.

Acknowledgements

We thank Prof. Alice Ting (MIT) for DNA plasmids for EGFR-BLAP and bacterial biotin ligase and Dr. Byron Ballou (CMU) for Cholera toxin-Qdots. This work was funded by the Villum Kann Rasmussen Foundation (BioNET), Leo Pharma Forskningsfond, the Lundbeck Foundation and the Novo Nordisk Foundation.