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# QUANTUM DOT CONJUGATIONS AND MULTICOLOR IMAGING IN LIVING CELLS

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## ABSTRACT

Quantum dots (Qdots) are fluorescent nanocrystals with a very high signal intensity and signal stability as compared to conventional fluorescent probes. Qdots are tunable in core size and thereby in emission wavelengths, while still having the same excitation wavelength. Furthermore Qdots are available with either primary amines or carboxyl groups on the surface hence allowing for easy covalent conjugation of essentially any biomolecule of interest to the Qdots.

We have exploited the photophysics of Qdots and elucidated and found ways to suppress the blinking and bleaching of Qdots. Taking advantage of these facts, we have designed and made Qdots with different biomolecule substrates conjugated to the surfaces targeting specific native or artificial tags on plasma membrane components. By applying bioconjugated Qdots of different colors in living mammalian cells, we are able to follow up to four different molecules at the single molecule level at the same time at millisecond integration times. Doing this, we are able to study spatial and temporal organization of various combinations of lipids and proteins in the plasma membrane.

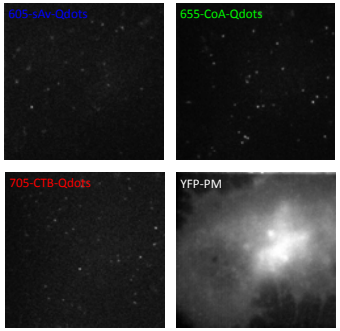
Specifically, we have conjugated Co-enzyme A (CoA) on to pegylated amino Qdots reacting the primary amine with the sulfhydryl on the CoA moiety via a crosslinker molecule. CoA targets the Acyl Carrier Protein tag (ACP-tag) which can be expressed as a fusion protein in the outer leaflet of the plasma membrane exposing the ACP-tag to the substrate CoA-Qdots. The data are analysed using k-space image correlation spectroscopy (kICS) programs developed by Paul Wiseman and his group[1].

## MULTICOLOR SINGLE PARTICLE TRACKING (SPT) OF PROTEINS AND LIPIDS

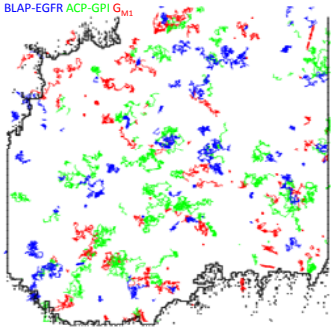
Our goal is now to implement and apply the 655-CoA-Qdots (emitting at 655 nm) targeting the ACP-GPI together with other targeting systems in order to investigate the nano-organization of the cellular plasma membrane by high-speed, multicolor, simultaneous SPT of a combination of membrane proteins and 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 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, to visualize the plasma membrane.

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 25 Hz and in the presence of 200 μM DTT to prevent Qdot color shifting and minimize blinking.



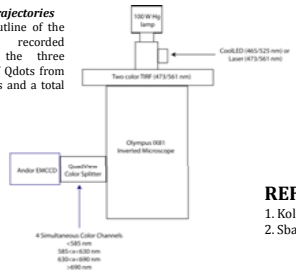

***Snap-Shots From the Four Different Recording Channels.***
*Upper left:* 605-sAv-Qdots which target biotinylated tyrosine kinase receptor, BLAP-EGFR.
*Upper right:* 655-CoA-Qdots which target GPI-anchored ACP.
*Lower left:* 705-CTB-Qdots which target ganglioside $G_{M1}$.
*Lower right:* Sum of all frames in the YFP channel.




***Single Particle Trajectories***
Overlay of the outline of the cell and the recorded trajectories of the three different kinds of Qdots from 567 image frames and a total duration of 22.8 s.

## 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.




## k-SPACE CORRELATION SPECTROSCOPY

**Image correlation techniques**, such as temporal image correlation spectroscopy (TICS) and the more generalized spatio-temporal image correlation spectroscopy (STICS), can be used to analyze a series of fluorescence images taken over time to extract ensemble-averaged information about molecular motions, such as:

- Diffusion coefficient
- Flow speed
- Aggregation state of labeled molecules
- Density of labeled molecules
- Immobile fraction

These techniques can be used with Qdots as labels, but blinking of the Qdot labels can lead to inaccurate results.

**kICS** is a newly developed technique that can determine many of the same parameters from a simple fluorescence image series but is completely unaffected by blinking of the fluorophores[1]. The schematic below shows how kICS works.

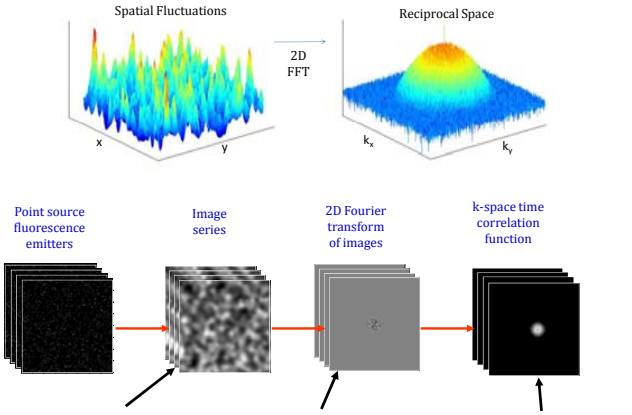



Image collected is a convolution of the point-source fluorophore positions $\rho(\mathbf{r}, t)$ with the point spread function $I(\mathbf{r})$ of the microscope.

A 2D FFT transforms each image to its k-space representation.

The average autocorrelation is determined at each **k** value for a range of time lags **τ**.

### The k-space time correlation function

Decay of the k-space autocorrelation function with time is fit to an analytical model that includes **flow**, or **diffusion**, or both.

$$r(\vec{k},\tau) = N \frac{q^2 I_0^2 \omega_0^2 \pi}{4} \left\langle \Theta(t)\Theta(t+\tau) \right\rangle \exp\left[ i\vec{k}\cdot\vec{v}\tau - |\vec{k}|^2 \left( D\tau + \frac{\omega_0^2}{4} \right) \right]$$

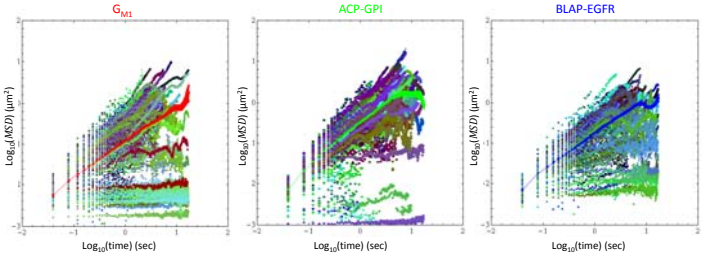
**Photophysical fluctuations** such as blinking or bleaching or fluorophores can be separated from transport (diffusion or flow) in a plot of **ln(r)** vs. values of k or $k^2$. (This requires the assumption that photophysical fluctuations are independent of transport.)

**N** = number of particles
**q** = constant including collection efficiency of the microscope, quantum yield, etc.
$\mathbf{I_0}$ = incident laser intensity
$\boldsymbol{\omega_0^2} = e^{-2}$ gaussian beam radius
**Θ(t)** = 1 if fluorophore is emitting at time t, otherwise 0
**v** = flow velocity
**τ** = time lag between images being correlated
**D** = diffusion coefficient

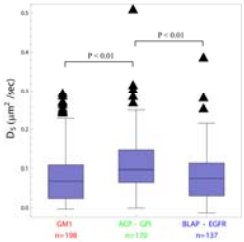
## REFERENCES

1. Kolin et al. Biophysical Journal 91 3061-3075 (2006)
2. Sbalzarini, I.F. & Koumoutsakos, P., J. Struct. Biol. 151, 182-195, (2005)

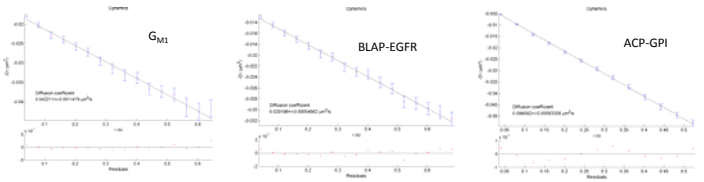
## RESULTS FROM SPT ANALYSIS




***SPT Diffusion Data From the Three Membrane Species.***
Single particle tracking (SPT) data was analyzed by use of a Particle Tracker plug-in in ImageJ[2] and by post-processing in Mathematica.
*Upper panel:* log(*MSD*) vs. log(*time*) of detected trajectories (length > 10 image frames) of the three investigated molecular membrane species: BLAP-EGFR, ACP-GPI, and $G_{M1}$. In all cases a lot of heterogeneity is observed and a wide range of migration behavior is seen. Marked by pure blue, green, and red, respectively is the *MSD* of the average of all trajectories.
*Lower right:* The heterogeneity is also seen in the box- and whiskers plot of the diffusion coefficients, $D_5$, from fitting the first five points to normal diffusion (MSD=4Dt), of the individual particle trajectories of the three molecular species. Outliers are shown as triangles.




## RESULTS FROM kICS ANALYSIS




## SUMMARY OF RESULTS

| Membrane Molecule \ Analysis method | Average Diffusion, SPT: Average trajectory analyses | Average Diffusion, SPT: Single trajectory analyses | Average Diffusion, kICS: Correlation analyses |
|---|---|---|---|
| | $D$ (μm²/s) +/- A.S.E | $D$ (μm²/s) +/- STD | $D$ (μm²/s) +/- STD |
| **BLAP-EGFR** | 0.053 +/-0.002 | 0.068 +/-0.063 | 0.029 +/- 0.001 |
| ACP-GPI | 0.078 +/-0.008 | 0.106 +/-0.081 | 0.099+/- 0.001 |
| GM1 | 0.038 +/-0.001 | 0.076 +/- 0.066 | 0.042 +/- 0.001 |

## CONCLUSIONS

The preliminary results from the kICS analyses are in the case of ACP-GPI and $G_{M1}$ in very close agreement with the average trajectory SPT MSD analyses (1st and 3rd columns in Table) and in the case of ACP-GPI, also with the single trajectory MSD analysis (2nd and 3rd columns in Table). The agreement is less in the case of the average trajectory analyses of BLAP-EGFR, and in the case of the single trajectory analyses of $G_{M1}$ and BLAP-EGFR. This discrepancy is likely due to differences in the area of interest and length of the analyzed image sequences between the SPT and kICS analysis, and most certainly also to the greater observed heterogeneity in the particle trajectories of $G_{M1}$ and BLAP-EGFR as compared to ACP-GPI. The large observed heterogeneity in the particle trajectories is also the reason for the discrepancy in the results from the average trajectory versus the single trajectory SPT analyses.

## ACKNOWLEDGEMENTS

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