

Self-Controlled Monofunctionalization of Quantum Dots for Multiplexed Protein Tracking in Live Cells**

Changjiang You, Stephan Wilmes, Oliver Beutel, Sara Löchte, Yulia Podoplelowa, Friedrich Roder, Christian Richter, Thomas Seine, Dirk Schaible, Gilles Uzé, Samuel Clarke, Fabien Pinaud, Maxime Dahan, and Jacob Piehler*

Tracking the motion of individual proteins on the surface of live cells has contributed considerably towards unveiling the functional organization of proteins in the plasma membrane.^[1] Individual proteins labeled with quantum dots (QDs) can be imaged over long time periods with ultrahigh spatial and temporal resolution, yielding powerful information on the spatiotemporal dynamics of proteins at the plasma membrane in live cells.^[2–7] A key challenge for the application of QDs is to site-specifically attach proteins to the surface of these nanoparticles in a stoichiometric manner without affecting protein function. Several procedures for rendering surfaces of QDs biocompatible have been described,^[4,7–12] thus reducing non-specific binding and protein denaturation on the QD surface. However, functionalized biocompatible QDs used to target cell surface proteins generally result in multipoint attachment to the target proteins because the number of functional groups on the QDs is very difficult to control. Such multiply functionalized QDs induce clustering of target proteins on the cell surface, biasing not only lateral diffusion but also the functional properties of these proteins. As a consequence, increased endocytosis has been observed upon binding of QDs functionalized with multiple epidermal growth factor (EGF) molecules to cell-surface EGF receptors.^[3] Stochastic functionalization of multiple reactive sites on the QD offers the choice of obtaining only a minor fraction of the QDs with a single functional group,^[3,13] or a significant

fraction of QDs with multiple functional groups. Preparation of homogeneous, monofunctional QDs currently relies on electrophoretic purification, which has been achieved only for very small QDs. These compact QDs are designed with very thin surface coatings,^[14] which have the disadvantage of showing relatively strong non-specific interactions.

Most approaches for targeting proteins using QDs in live cells are based on biotin–streptavidin interactions, which form quasi-irreversible complexes. For multiplexed, generic labeling of proteins on the cell surface, further targeting strategies are required. We have recently described tris(hydroxymethyl)methylamine–nitrilotriacetic acid (Tris-NTA) moieties for highly specific and stable attachment of fluorophores and other functional units to histidine-tagged proteins in vitro and on the surface of live cells.^[15–17] The lifetime of Tris-NTA complexes with His-tagged proteins is in the order of several hours, which is well-suited to medium-term single molecule tracking applications. Herein, we have attempted to control the functionalization degree of QDs with Tris-NTA by means of electrostatic repulsion. We devised a bottom-up coupling chemistry based on a novel Tris-NTA derivative (**1**; Figure 1a), which comprises a thiol-terminated hepta(ethylene glycol) linker. This compound was generated in situ by reduction of the disulfide-linked dimer (**1a**; see the Supporting Information, Scheme S1) and coupled to commercially available polymer-coated and amine-functionalized QDs by means of a hetero-bifunctional cross-linker (Figure 1b). Covalently attachment of **1** to surfaces modified with maleimide-functionalized polyethylene glycol (PEG) polymer brush and specific immobilization of His-tagged proteins was confirmed by label-free detection (Supporting Information, Figure S1). To control the degree of functionalization with Tris-NTA on the QD surface, the reaction of **1** with surface maleimide groups was performed at low ionic strength. Under these conditions, all QDs were reacted with Tris-NTA, as confirmed by an increase in negative charges detected by anion exchange chromatography and agarose gel electrophoreses (Figure 1c,d). These assays indicated relatively monodisperse electrostatic properties after coupling of **1**, despite the fact that it was reacted at a large excess (660 μM of compound **1** to 1 μM QD). Coupling of **1** at higher ionic strength yielded QDs with a substantially higher degree of functionalization, as confirmed by a further shift of the signals both in anion exchange chromatography and agarose gel electrophoresis (Figure 1c,d).

To characterize the functional properties of Tris-NTA-coupled QDs, binding to immobilized hexahistidine (H6)

[*] Dr. C. You, S. Wilmes, O. Beutel, S. Löchte, Y. Podoplelowa, F. Roder, C. Richter, T. Seine, D. Schaible, Prof. Dr. J. Piehler
Division of Biophysics, Universität Osnabrück
Barbarstrasse 11, 49076 Osnabrück (Germany)
Fax: (+49) 541-969-2262
E-mail: piehler@uos.de
Homepage: <http://www.biologie.uni-osnabrueck.de/Biophysik/Piehler/>

Dr. G. Uzé
CNRS UMR 5124, University Montpellier II
Place Eugène Bataillon, Montpellier (France)
Dr. S. Clarke, Dr. F. Pinaud, Dr. M. Dahan
Département de Physique et de Biologie
Ecole Normale Supérieure, Paris (France)

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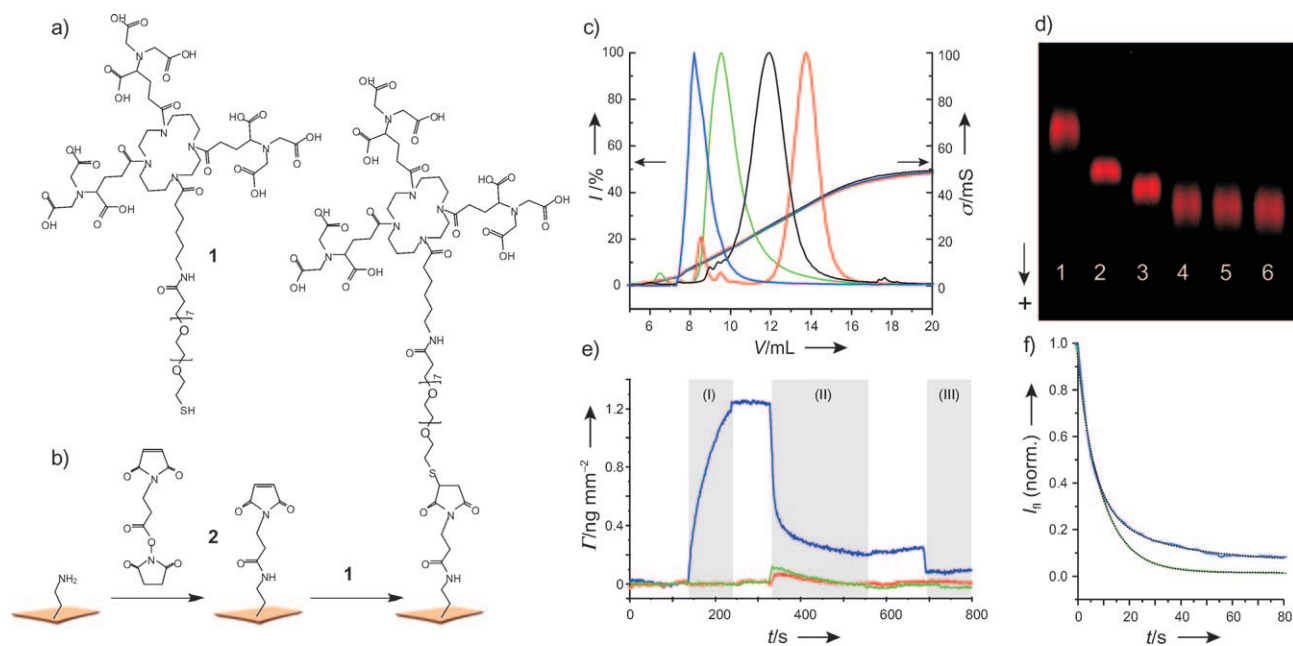


Figure 1. Characterization of Tris-NTA-functionalized QDs in vitro. a) Structure of the Tris-NTA derivative **1** used for surface functionalization. b) Surface amines were treated with 3-maleimidopropionic acid-*N*-hydroxysuccinimidyl ester (MPA-NHS; **2**), and then Tris-NTA-EG₇-thiol **1** was coupled. c) Fractionation by anion-exchange chromatography: elution profiles (left y axis) and conductivity (right y axis) of amine-functionalized QDs (blue), after reaction with MPA-NHS (green), and coupling of **1** at low (black) and high (red) ionic strength. d) Agarose gel electrophoreses of QD before functionalization (lane 1), after reaction with MPA-NHS (**2**), and after coupling of Tris-NTA at different NaCl concentrations: 3) 0 mM, 4) 150 mM, 5) 400 mM, 6) 1 M. e) Binding of nickel(II)-loaded QD⁶⁵⁵-Tris-NTA to a H6 peptide surface and elution with 250 mM imidazole (blue) compared to unmodified QD⁶⁵⁵ (red) and QD⁶⁵⁵-Tris-NTA incubated with 1 mM EDTA (green). I) Injection of QDs, II) elution with 250 mM imidazole, III) regeneration with 100 mM HCl. f) Comparison of the dissociation kinetics for QD⁶⁵⁵-Tris-NTA (blue) and OG⁴⁸⁸-Tris-NTA (green) from immobilized H6 peptide in the presence of 125 mM imidazole.

peptides was probed by simultaneous total-internal-reflection fluorescence spectroscopy and reflectance–interference (TIRFS-RIF) detection. Fully specific binding was observed only after loading the NTA groups with nickel(II) ions (Figure 1 e), thus confirming successful functionalization and selective capturing of His tags by the Tris-NTA moiety. Selective elution with the His-tag competitor imidazole confirmed nickel(II)-mediated binding. We could not detect binding of QDs without Tris-NTA, corroborating efficient shielding of the ZnS surface, which can non-reversibly interact with His-tagged proteins.^[18] Upon elution with imidazole, very similar dissociation kinetics compared to Oregon Green 488-labeled Tris-NTA (OG⁴⁸⁸-Tris-NTA) was observed (Figure 1 f; $k_d = 0.073 \text{ s}^{-1}$ and 0.10 s^{-1} for QD⁶⁵⁵-Tris-NTA and OG⁴⁸⁸-Tris-NTA, respectively), confirming a low degree of Tris-NTA functionalization in the case of coupling at low ionic strength.

Specific protein binding to QD⁶⁵⁵-Tris-NTA was explored by analytical size-exclusion chromatography (SEC). For this purpose, nickel(II)-loaded QD⁶⁵⁵-Tris-NTA (10 nM) were incubated with a large excess (1 μM) of His-tagged maltose-binding protein (MBP-H10) labeled with a Cy5 analogue dye (FEW⁶⁴⁶-MBP-H10). QD-bound FEW⁶⁴⁶-MBP-H10 was separated from the free protein by SEC using spectral detection for photometric quantification of QD⁶⁵⁵-Tris-NTA and FEW⁶⁴⁶-MBP-H10 (Supporting Information, Figure S2). These assays confirmed specific nickel(II)-mediated binding of His-tagged

protein to the QD surface. The protein/QD ratio was estimated from the signals at 350 nm (QD⁶⁵⁵-Tris-NTA) and 650 nm (QD⁶⁵⁵-Tris-NTA + FEW⁶⁴⁶-MBP-H10). From these measurements, a protein/QD ratio of 0.9:1 was determined, suggesting an average degree of functionalization of about unity. In conjunction with the observation that all QDs were reacted with Tris-NTA (quantitative shift in anion-exchange chromatography), this result strongly points towards a monodisperse 1:1 functionalization. This observation suggests that electrostatic repulsion may steer the reaction: once a nanoparticle is conjugated with the first Tris-NTA molecule, its highly negative electrostatic potential (six negative charges) efficiently shields coupling of a second Tris-NTA. This effect is maximized by using very low ionic strength and by keeping the NTA moieties free of transition metal ions.

The functional properties of proteins captured to QDs were explored by quantitative protein–protein interaction analysis. For this purpose, we produced Interferon- $\alpha 2$ (IFN $\alpha 2$) fused to an N-terminal decahistidine tag (H10-IFN $\alpha 2$). Nickel(II)-loaded QD⁶⁵⁵-Tris-NTA was incubated with a large excess of H10-IFN $\alpha 2$, followed by purification of the QD⁶⁵⁵-Tris-NTA/H10-IFN $\alpha 2$ complex by SEC. Binding of QD⁶⁵⁵-Tris-NTA/H10-IFN $\alpha 2$ to the extracellular domain of its receptor subunit IFNAR2 (IFNAR2-EC) tethered to solid-supported membranes through a C-terminal H10-tag (Figure 2 a) was probed by TIRFS-RIF (Figure 2 b). Specific binding to the membrane-bound IFNAR2-EC was observed

(Figure 2c), followed by dissociation from the surface during elution with imidazole. The dissociation rate constant $k_d = 0.028 \pm 0.003 \text{ s}^{-1}$ determined from the binding curve is only slightly higher than the dissociation rate constant observed for non-labeled IFN α 2 (0.015 s^{-1}) and the dissociation rate constant of H10-IFN α 2 labeled with OG488 Tris-NTA ($0.018 \pm 0.002 \text{ s}^{-1}$; Supporting Information, Figure S3). In a control

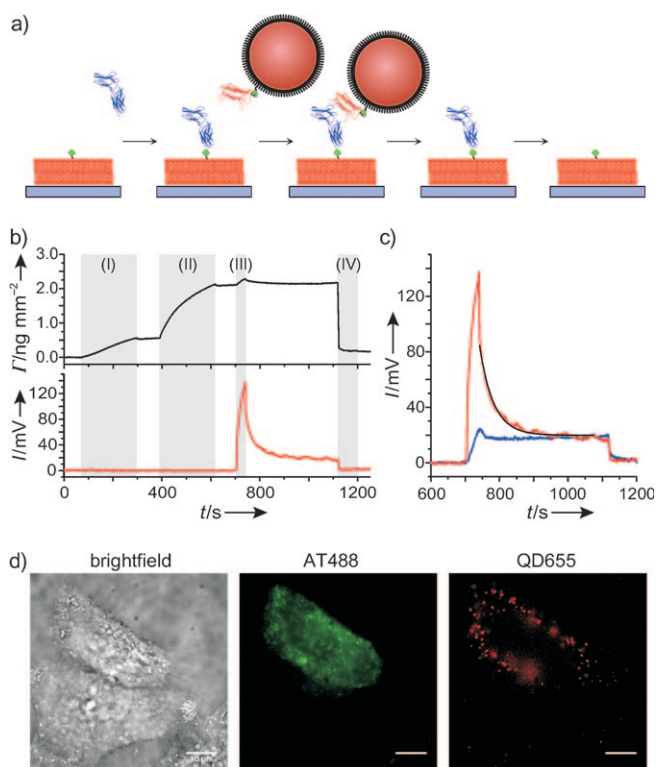


Figure 2. Site-specific protein labeling with QD655 Tris-NTA. a) Tethering of IFNAR2-EC (blue) onto a solid-supported membrane, binding and dissociation of QD655 Tris-NTA/H10-IFN α 2 (red), and regeneration with imidazole. b) Binding of QD655 Tris-NTA/H10-IFN α 2 to IFNAR2-EC tethered on solid-supported membranes as detected by simultaneous RIF (top) and TIRFS detection (bottom). Gray bars: injection periods for IFNAR2-EC (I), MBP-H10 (II), QD655 Tris-NTA/H10-IFN α 2 (III), and imidazole (IV). c) Binding kinetics of QD655 Tris-NTA/H10-IFN α 2. As a negative control, binding of QD655 Tris-NTA without H10-IFN α 2 to IFNAR2-EC tethered membranes was measured (blue). d) Specific binding of QD655 Tris-NTA/H10-IFN α 2 to HeLa cells expressing IFNAR2- Δ IC. Strong binding of AT488 IFN α 2 to the same cell is shown for comparison. Scale bars: 10 μm .

experiment, the dissociation rate constant of IFNAR2-EC from H10-IFN α 2 immobilized on a PEG polymer brush was determined by TIRFS detection (Supporting Information, Figure S3), yielding a very similar dissociation rate constant ($0.031 \pm 0.001 \text{ s}^{-1}$) as that obtained for QD655 Tris-NTA/H10-IFN α 2 binding to immobilized IFNAR2-H10. Importantly, the unbiased dissociation kinetics confirmed the monovalent functionalization of the QDs with Tris-NTA: for multivalent QDs obtained by Tris-NTA coupling at high ionic strength, no

ligand dissociation from the surface was observed, as expected for multiple IFN α 2 molecules bound to the QD (Supporting Information, Figure S3).

In the next step, we explored binding of IFN α 2 labeled with QD655 Tris-NTA to IFNAR2 in live cells. For this purpose, HeLa cells were transfected with a plasmid coding for IFNAR2, which was truncated after the transmembrane domain (IFNAR2- Δ IC).^[16] Specific binding of IFN α 2 labeled with ATTO 488 (AT488 IFN α 2) only to cells overexpressing of IFNAR2- Δ IC at the cell surface was used to detect transfected cells (Figure 2d). Upon incubation of QD655 Tris-NTA/H10-IFN α 2, binding only to the cells expressing IFNAR2- Δ IC was observed, confirming specific ligand binding. High mobility of QD655 Tris-NTA/IFN α 2 on the cell surface was observed by single-molecule tracking. We analyzed the diffusion by single molecule tracking for both AT488 IFN α 2 and QD655 Tris-NTA/IFN α 2 bound to IFNAR2- Δ IC (Supporting Information, Figure S4). From the mean-square displacement (MSD) analysis, diffusion constants of $0.10 \pm 0.02 \mu\text{m}^2 \text{ s}^{-1}$ and $0.066 \pm 0.003 \mu\text{m}^2 \text{ s}^{-1}$ were obtained for the mobile fraction of AT488 IFN α 2 and QD655 Tris-NTA/IFN α 2, respectively ($52 \pm 10 \%$ and $46 \pm 2 \%$, respectively, of all tracked particles).

We have previously demonstrated the high selectivity of Tris-NTA enabling for targeting fluorescence probes to proteins on the cell surface.^[15,16] We therefore explored direct labeling of a His-tagged protein on the cell surface with QD655 Tris-NTA. For this purpose, membrane-anchored CFP fused to an N-terminal H10-tag (H10-CFP-TMD) was expressed in HeLa cells and incubated with nickel(II)-loaded QD655 Tris-NTA (Figure 3a). Binding of QD655 Tris-NTA to the cell surface correlated very well with the cell surface expression level of H10-CFP-TMD as detected in the CFP channel. QD655 Tris-NTA was quantitatively removed from the cell surface by washing with imidazole (Supporting Information, Figure S5), confirming nickel(II)-NTA-mediated binding to the cell surface. High mobility of QDs bound to H10-CFP-TMD was observed (Supporting Information, Figure S6).

The diffusion constant and the mobile fraction determined by single molecule tracking was $0.095 \pm 0.005 \mu\text{m}^2 \text{ s}^{-1}$ and $49 \pm 2 \%$, respectively, corresponding well to the parameters obtained by FRAP ($0.10 \pm 0.02 \mu\text{m}^2 \text{ s}^{-1}$ and $52 \pm 1 \%$; Supporting Information, Figure S6). This finding corroborates with unbiased individual protein labeling with QD Tris-NTA on the cell surface. With these tools in hand, we combined targeting of cell surface protein and functional labeling of protein in vitro into a dual-color experiment. For this purpose, full-length IFNAR2 fused to an N-terminal H10 (H10-IFNAR2) was first directly labeled with QD525 Tris-NTA, followed by binding of QD655 Tris-NTA/IFN α 2. Staining with QD525 Tris-NTA was only observed for cells, which also showed binding of QD655 Tris-NTA/IFN α 2, confirming labeling specificity. No stable co-localization of QD655 Tris-NTA and QD525 Tris-NTA was observed, which may be ascribed to the fact that only a small fraction of IFNAR2 on the surface was labeled with QDs ($< 5 \%$), resulting into a very low probability for co-localization. This experiment enables to analyze and compare diffusion of IFNAR2 with and without ligand in the same cell.

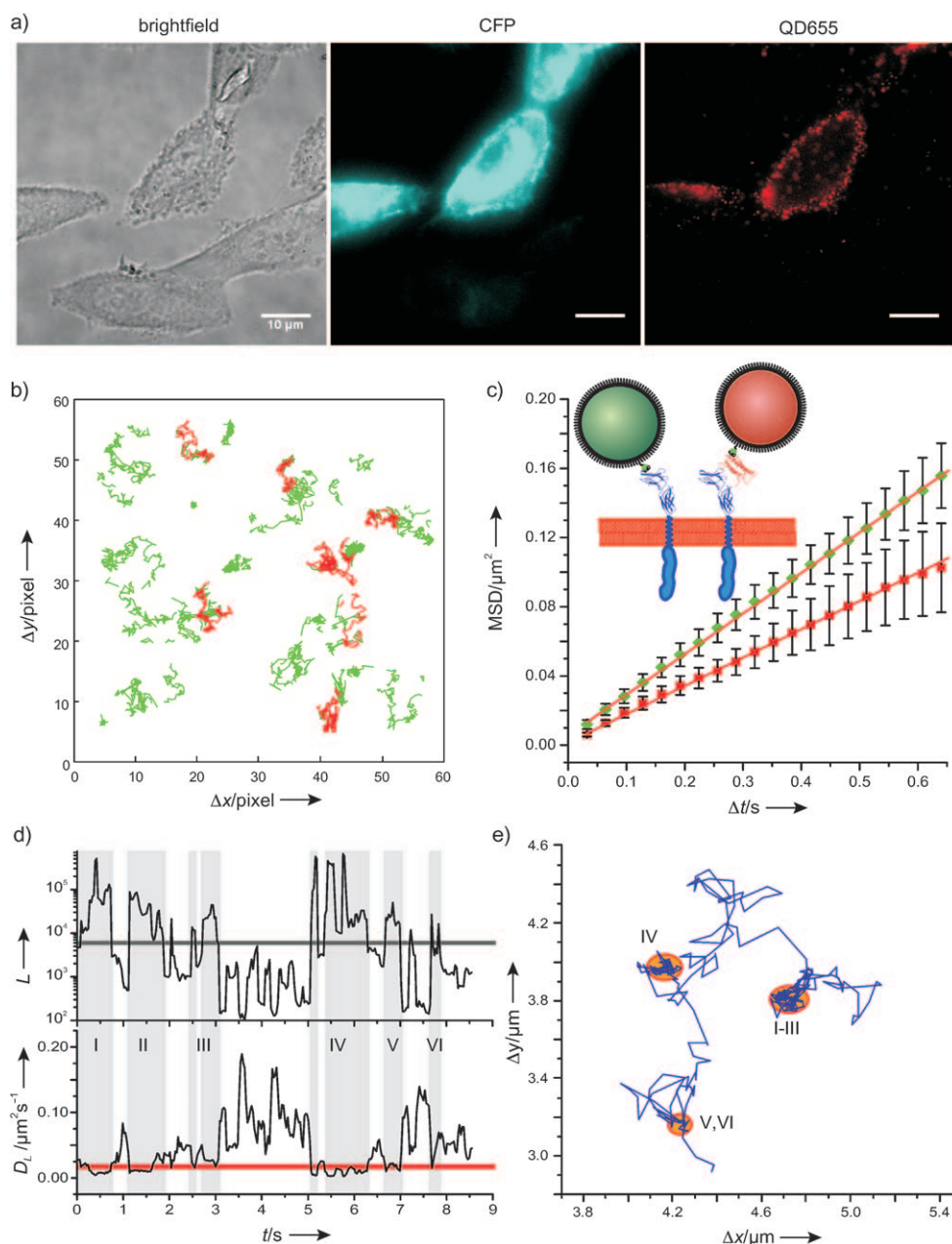


Figure 3. QD^{655} Tris-NTA-labeled IFN α 2 binding and tracking in live cells. a) Comparison of a brightfield image and fluorescence images (CFP and QD655 channels) of cells transiently transfected with H10-CFP-TMD and incubated with nickel(II)-loaded QD^{655} Tris-NTA. Scale bars: 10 μ m. b) Trajectories obtained for simultaneous tracking of H10-IFNAR2 in live cells labeled with QD^{525} Tris-NTA (green) and QD^{655} Tris-NTA/H10-IFN α 2 (red). c) MSD analysis of QD^{525} Tris-NTA/H10-IFNAR2 and QD^{655} Tris-NTA/H10-IFN α 2 in live cells. d) Analysis of a single trajectory obtained for QD^{655} Tris-NTA/H10-IFN α 2 showing the local diffusion constant (D_L , bottom) and the confinement factor L (top) using an evaluation window of five frames. Sections of the trajectory identified as confined diffusion are highlighted in gray. e) Same trajectory with the confined regions indicated in orange.

Single-molecule tracking of QD^{655} Tris-NTA and QD^{525} Tris-NTA, and standard MSD analysis, yielded diffusion constants of $0.038 \pm 0.003 \mu\text{m}^2\text{s}^{-1}$ for the IFN α 2-IFNAR2 complex and $0.058 \pm 0.002 \mu\text{m}^2\text{s}^{-1}$ for free IFNAR2 alone (Figure 3b,c). Slower diffusion of ligand-bound IFNAR2 may be ascribed to

ternary complex formation with endogenous IFNAR1, which is present at relatively low level (ca. 200 receptors/cell).

In vitro binding studies with IFNAR1-H10 and IFNAR2-H10 tethered onto solid-supported membranes confirmed that ternary complex formation upon QD^{655} Tris-NTA/IFN α 2 binding was possible (Supporting Information, Figure S7). Single-trajectory analysis of the QD^{655} Tris-NTA-labeled IFN α 2-IFNAR2 complex with respect to local confinement^[19] revealed, however, periods of relatively fast diffusion (ca. $0.1 \mu\text{m}^2\text{s}^{-1}$) alternating with periods during which very little mobility can be observed (Figure 3d,e and the Supporting Information). The exact reasons for this behavior need to be explored in the future. Similar events have been previously described, for example, for the EGFR receptor.^[19]

In summary, we have demonstrated successful monofunctionalization of QDs with Tris-NTA for selectively capturing His-tagged proteins in vitro and in live cells with a well controlled 1:1 stoichiometry. Our results confirm that electrostatic steering controls nanoparticle functionalization to a 1:1 ratio, in contrast to the Poisson distribution obtained by standard functionalization techniques. Monovalent Tris-NTA QDs have versatile applications for cell surface labeling for unbiased single-molecule tracking: they can not only be directly targeted to His-tagged proteins on the cell surface, but they can also be readily conjugated with

any other His-tagged recognition unit, such as monovalent streptavidin^[20] or recombinant single-chain antibody fragments in a stoichiometric manner. Indeed, we could also conjugate His-tagged monovalent streptavidin with QD^{655} Tris-NTAs for live-cell labeling and tracking of IFNAR1, which

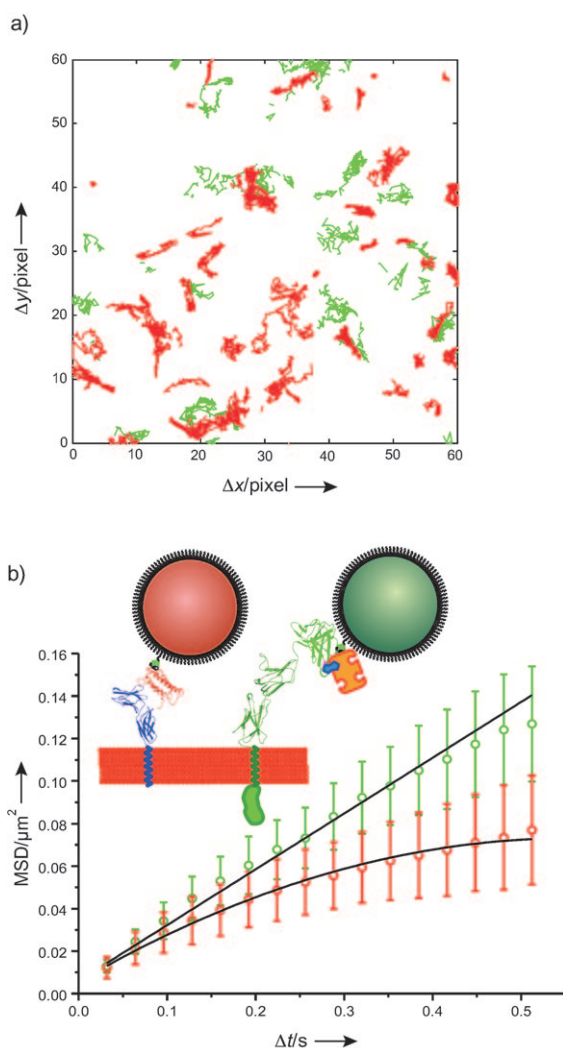


Figure 4. Simultaneous tracking of H10-IFN α 2/QD⁶⁵⁵-Tris-NTA bound to cell-surface IFNAR2- Δ IC and H6-mSAv/QD⁵²⁵-Tris-NTA targeted to biotinylated AP-IFNAR1. a) Trajectories obtained for H6-mSAv/QD⁵²⁵-Tris-NTA/AP-IFNAR1 (green) and for QD⁶⁵⁵-Tris-NTA/H10-IFN α 2 (red). Image dimensions: 10.8 μ m $\times$ 10.8 μ m. b) Mean-square displacement analysis of H6-mSAv/QD⁵²⁵-Tris-NTA bound to AP-IFNAR1 (green) and QD⁶⁵⁵-Tris-NTA/H10-IFN α 2 (red) in live cells. A diffusion constant of $0.066 \pm 0.001 \mu\text{m}^2 \text{s}^{-1}$ and a mobile fraction of $32 \pm 3\%$ were obtained for IFNAR1 (green), whilst anomalous diffusion was observed for QD⁶⁵⁵-Tris-NTA/H10-IFN α 2 (red).

was site-specifically biotinylated through an N-terminal AP tag (Figure 4). Our approach is compatible with most coatings, including those that are commercially available, and

should thus provide generic access to monovalent nanoparticle biofunctionalization.

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