Supporting information

A small peptide-protein interaction pair for genetically encoded, fixation compatible peptide-PAINT

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Materials and methods

DNA constructs

The amino acid sequences for c-terminal peptideDTWV and the VHHmCherry, PDZ and ePDZb1 domains are given in Table S1.

Vimentin-mCherry, Vimentin-mCherry-peptideDTWV and TOMM20-mCherry-peptideDTWV: for expression in COS7 cells: TOMM20 and the codon optimized vimentin sequence flanked by AgeI and EcoRI restriction sites were ordered as gBlock from IDT. mCherry with or without the c-terminal peptide and flanked by EcoRI and BamHI was amplified by PCR. After restriction, the inserts were ligated in an AgeI/BamHI linearized pEGFP-C1 backbone.

PDZ-mNG and ePDZb1-mNG: the Erbin PDZ domain1,2 (PDZ) and the enhanced PDZb1 affinity clamp3 (ePDZb1) sequences flanked by BamHI (N-terminus) and HindIII (C-terminus) were amplified by PCR. mNeonGreen was amplified with a N-terminal GSGS linker and HindIII domain and a C-terminal XhoI domain. After restriction the fragments were ligated in a linearized pET-28a expression vector to generate PDZ-mNG and ePDZb1-mNG followed by a 6xHis-tag for purification.

VHHmCherry-peptideDTWV: The Lam4 nanobody sequence4, codon optimized for bacterial expression, was ordered as gBlock from IDT with a N-terminal his-tag, C-terminal linker-peptideDTWV sequence and flanking NcoI and XhoI restriction sites (table S1, VHHmCherry-peptideDTWV). The restricted insert was ligated in the pET-28a expression vector that was linearized with the corresponding restriction enzymes. All sequences were verified by sequencing before transfection or transformation into the BL21 DE3 expression strain.

protein purification

Protein expression was performed in BL21 DE3 bacterial cells, grown in LB broth supplemented with 50 µg/mL kanamycin, transformed with the constructs in the pET28a vector described above. Expression was induced at OD0.6 with IPTG and cells were grown for 0.5 hours at 37°C followed by overnight shaking at 20°C. After induction, bacteria were pelleted by centrifugation and the supernatant was discarded. The bacterial pellet was kept on ice and resuspended in PBS supplemented with EDTA-free protease inhibitor cocktail (Sigma) and lysozyme. After 15 minutes the cells were sonicated in 6 rounds (40 seconds followed by 30 seconds rest). The soluble fraction was then separated from the insoluble fraction by centrifugation (10,000x g for 45 min) and incubated with PBS washed Ni-NTA beads for 1 hour at 4°C. The beads containing the proteins were then collected in a disposable column (Bio-rad) and washed two times with 15 mL PBS, followed by two washes with PBS + 40mM Imidazole. The protein was then eluted by incubation with PBS + 400mM Imidazole. Finally, buffer exchange to PBS was performed (PD-10 desalting columns, Cytiva) and the protein was supplemented with 10% (w/v) glycerol and flash frozen in liquid nitrogen. Protein concentrations were determined by SDS-page of the purified proteins next to a BSA standard. The final concentrations were for 2.69, 9.97 and 117 µM for PDZ-mNG, ePDZb1-mNG and VHHmCherry-peptideDTWV respectively.

Preparation of polystyrene nanoparticles labelled with peptide

The biotin-Aminohexanoic acid-GAGRSIDTWV peptide (biotin-peptideDTWV) sequence was ordered from Genscript and dissolved in milli-Q water to 1mM final concentration. The peptide solution was stored at -20°C. Labelling of 300 nanometer streptavidin-coated polystyrene particles (Spherotech, SVP-03-10) was performed by mixing 15µl beads (10m/mL stock) with 10 µl peptide and 125 µl PBS in Protein LoBind Eppendorf tubes (Eppendorf, EP0030108116), followed by 20 minutes sonication and 1.5 hour shaking at room temperature. The beads were then washed three times by pelleting through centrifugation (15000x g, 4°C), aspiration of the supernatant and resuspension in 300 µL PBS. After the
final wash, the beads were resuspended in 150 µL PBS so that the final concentration was 1mg/mL and then stored at 4°C.

**Microscopy slide passivation and sample preparation for peptide-PAINT on polystyrene beads and qPAINT calibration**

**Coverslip passivation and functionalization.** Coverslips (Thermo Scientific Menzel x1000, #1.5, 22x22mm) were sonicated for 10 minutes in methanol and air dried with nitrogen gas. Subsequently, the coverslips were plasma cleaned (plasmatreat, FG5001) for 1 minute and stored in a closed container. Cleaned coverslips were used within 8 hours of cleaning. Coverslips were mounted on a microscopy slide spaced by double sided tape to generate a channel. The plasma treated surface in the channels were functionalized for 10-15 minutes with 1mg/mL PLL-PEG/PLL-PEG-biotin mixed in an equimolar ratio (Susos AG, Switzerland) and subsequently washed three times by flushing 100ul PBS through the channel.

**Single molecule localization microscopy on polystyrene beads decorated with biotin-peptide coated beads** (final concentration 0.1mg/mL) and the PDZ-mNeonGreen probe (concentrations are specified in table S2) were diluted in PBS supplemented with 2% BSA to block aspecific interactions. Two chamber volumes (~20 µL) of the sample were then flushed into the above described functionalized channels and the channel was sealed with super glue to avoid evaporation. Imaging was then performed as described below.

**qPAINT calibration on immobilized biotin-peptide** After functionalization with PLL-PEG-biotin and washing, the channels were incubated for 5 minutes with 0.025 mg/mL streptavidin (Thermo Scientific, 21135) diluted in 2%BSA in PBS. Channels were then washed three times with 100 µL PBS. Subsequently, 100 µL 2nM biotin-peptide dilution in PBS containing 2% BSA was flushed through the channel and incubated for 4 minutes. Samples were washed five times by flushing 100ul PBS through the channel. Finally 26.9nM PDZ-mNG and highly diluted 100 nanometer fiducial markers were diluted in PBS supplemented with 2% PBS and flushed into the channel that was sealed with super glue before imaging.

**Cell culture and transfections**

COS7 cells were cultured in glucose/glutamine containing DMEM (Gibco: 11-965-092) supplemented with 10% FCS and penicillin/streptomycin. To prepare COS7 cells for imaging they were plated in ibidi µ-Slide 8 Well Glass Bottom (IBIDI 80827) chambers one day before transfection. Transfection of 0.5µg plasmid DNA in 250 ul culture medium was performed using FuGENE HD transfection reagent (Promega) according to the manufacturer’s protocol. Cells were fixed and imaged one day after transfection.

**Cellular fixation and nanobody staining**

Cos7 cells plated on µ-Slides were washed two times with PBS prior to fixation. Subsequently the cells were incubated with fixation medium pre-warmed to 37°C for 10 minutes. For optimal structure preservation, fixation solution for cells that expressed vimentin consisted of 4% paraformaldehyde (PFA) + 0.5% triton-x in PBS6-7, TOMM20 expression constructs were fixed with 3%PFA +0.1% (v/v) glutaraldehyde in PBS8. After fixation, cells were washed 3x with PBS. PFA/GA fixed TOMM20 samples were then reduced with fresh 1mg/mL fresh sodium borohydrate in PBS solution for 4 minutes and washed 5 more times with PBS. After washing, all samples were permeabilized with 0.5% triton-x in PBS for 10 minutes. Cells were washed three more times in PBS and blocked for 30 minutes in blocking solution (2%BSA in PBS). Finally, for cells expressing the vimentin- and TOMM20-mCherry constructs that also encoded the peptide sequence, samples were mounted on the microscope and directly
imaged by dilution and resuspension of the PDZ-mNG into the blocking solution at the indicated concentration (Table S2).

For staining of vimentin-mCherry with the purified LaM4 nanobody (VHHmCherry), blocked samples were incubated with VHHmCherry diluted 1/100 in blocking solution for one hour. After three more washes in PBS, samples were incubated with blocking solutions for 30 minutes and imaged by directly diluting the probe in this solution at the indicated concentration in Table S2.

Confocal and Stimulated emission depletion (STED) microscopy

Confocal and STED microscopy of polystyrene nanoparticles and cells was performed on an Abberior Instrument Expert Line configuration equipped with a 100x NA 1.4 oil objective on an Olympus IX83 inverted microscope. Confocal acquisitions of mCherry and mNeonGreen were performed by excitation with the 488 and 561 nm lasers respectively. Subsequently, the pulsed 595 nm depletion beam was applied for STED of mNeonGreen. Samples were prepared as for SMLM described above, except the high affinity ePDZb1-mNG, instead of the transient PDZ domain, was now added into the 2% BSA in PBS blocking solution for high affinity binding at concentrations indicated in Table S2. The samples were then mounted and confocal and STED microscopy were performed in the presence of the bound and unbound ePDZb1-mNG. Images were processed in Fiji.

TIRF microscopy for SMLM

SMLM was performed on a Nikon Eclipse Ti-E N-STORM system equipped with a Nikon 100x Apo TIRF oil immersion objective (NA 1.49) and perfect focus system. Excitation was performed with the 488 nm laser (90 mW) within the MLC400B laser box (Agilent technologies) under TIRF or HiLo illumination through a quad-band polychroic mirror (Nikon 97335). An Ixon3 EMCCD (Andor) was used for detection, resulting in an effective pixel size of 160 nanometer. Astigmatism based three-dimensional acquisitions were performed using a cylindrical lens (Nikon) within the emission path. 3D calibration were obtained using tetraspec beads imaged with the 488 laser. The system was controlled using the Nikon NIS-elements software. Acquisitions were performed by diluting PDZ-mNG or ePDZb1-mNG in PBS+2% BSA to reduce background and according to the methods described above and the conditions in Table S2.

Detection and localization of SMLM acquisitions

Single molecule localization microscopy acquisitions were imported into ImageJ and detection and quantification were performed using the Detection of Molecules (DoM version 1.2.1, https://github.com/ekatriukha/DoM_Utrecht) plugin for ImageJ. To detect single molecules a minimal signal to noise ratio between 3 and 4 was chosen. For 3D acquisitions, the calibration was generated on the tetraspec beads in the experimental setup described above and the “make z calibration” function of DoM was used. Cross-correlation based drift-correction was performed. In the calibration for qPAINT, the tetraspec beads were used as fiducial markers. Images were then reconstructed in DoM except for the 3D rendering in figure 1c that was generated via Python code to plot the localizations of individual nanoparticles. The FRC resolution was calculated by splitting the localizations of 2D acquisitions every other 500 frames. The resulting reconstructions were then used as input in the Fourier Ring Correlation plugin for ImageJ to calculate the image resolution (threshold: fixed 1/7) for 3 different acquisitions.

Image and qPAINT analysis

For qPAINT analysis, a mean shift algorithm in Matlab was applied to the DoM localization tables in order to determine clusters of localizations corresponding to a single peptide or nanoparticle. For the
calibration, clusters were identified when at least 15 localizations were identified within a 100 nm diameter. For the 300 nm in diameter nanoparticles, the cutoff was set at 25 localizations within 500 nm. Prior to analysis of the bright and dark times, localizations in subsequent frames were linked and a gap of 3 frames was allowed before it was characterized as a new binding event. The average bright times of each event and dark times between event, per cluster were then extracted. To demonstrate the qPAINT counting potential of this interaction, the localizations from clusters of a known number of single peptOTWV molecules (2, 3, 5, 10 or 25) from the datasets of the calibration experiments were pooled and reanalyzed as if they corresponded to a single cluster. The resulting qPAINT-determined number could then be plotted against the observed number of molecules used. All graphs and fits were rendered and performed using GraphPad Prism 8.
Figure S1 (related to Figure 1). Negative control for single-molecule localization microscopy of immobilized nanoparticles decorated with biotinylated-peptDTWV. (a) SMLM acquisitions with 10nM PDZ-mNG of 300 nanometer streptavidin-coated nanoparticles incubated without (left) and with (right) biotin-peptDTWV. Inset shows brightfield (left) or widefield fluorescent microscopy (right) to localize the beads prior to SMLM imaging. (b) Zooms of the representative beads selected in (a). The contrast for the bead in the absence is enhanced 20 times to show the background signal. (c) Scatter plot for the number of localizations of randomly selected beads in (a) (n=10). Scale bar: 10µm (a), 500nm (b).
Figure S2 (related to Figure 1). One-step staining of pept_{DTWV} decorated nanoparticles with purified ePDZb1-mNG (a) Schematic representation for one-step detection of pept_{DTWV} via the ePDZb1 affinity clamp fused to mNeonGreen. (b) Confocal and corresponding STED image for 300 nanometer polystyrene nanoparticles decorated with pept_{DTWV} and detected with ePDZb1-mNG in a single field of view. Scale bar: 1µm.
Figure S3 (related to Figure 1 and 2). Characterization of PDZ-mNG binding kinetics on pept\textsubscript{DTWV} decorated nanoparticles (a) Schematic representation for the experimental setup to perform PAINT on polystyrene nanoparticles with the pept\textsubscript{DTWV}, PDZ-mNG interaction pair. (b) PAINT reconstruction with high concentrations of PDZ-mNG (10nM) for high resolution imaging. (c) PAINT reconstruction with low concentrations of PDZ-mNG (0.1nM) to distinguish and detect single binding events for qPAINT. (d) Number of localizations over time for the acquisition in (c). Scale bar: 500nm (b,c).
Figure S4 (related to Figure 2). Automated cluster assignment to characterize $\text{pept}_{\text{DTWV}}$/PDZ-mNG binding kinetics in qPAINT acquisitions. (a) Schematic representation for the experimental setup to generate a qPAINT calibration. Inset depict two examples of experimental localization clusters for single $\text{pept}_{\text{DTWV}}$ strands (left) and the corresponding results of the automatically assigned clusters. (b) Same dataset as in Fig. 2a-b but observed and detected clusters were pooled to determine the number of peptides (y-axis, mean ± s.d.) for a known number of observed clusters (x-axis, clustersize: 2, 3, 5, 10 and 25 with $n=500, 336, 200, 100, 40$ respectively). Dashed line indicates slope of 1 (c) Representative reconstruction of $\text{pept}_{\text{DTWV}}$ decorated nanoparticles in a qPAINT acquisition with PDZ-mNG and the corresponding results of the assigned clusters for further analysis. (d) Zoom of the region indicated in (c). (e) Normalized frequency distributions of the average dark times ($\tau_{d,*}$) per nanoparticle ($n=449$). Scale bar: $1\ \mu\text{m}$ (a,c), $5\ \mu\text{m}$ (b).
Figure S5 (related to Figure 3). Localization precisions and FRC image resolution obtained after single molecule acquisitions of cells expressing peptDWTWV. (a) Representative reconstruction of a 2D PAINT acquisition of 20,000 frames at 1nM PDZ-mNG concentration. (b) Plotted localization error (mean±sd) for three representative 2D PAINT acquisitions as shown in (a) (c) FRC plots for the three identical acquisitions as in (b) to determine the estimated FRC image resolution. Scale bar: 5µm.
Figure S6 (related to Figure 3). Negative control for single-molecule localization microscopy in cells expressing pept$_{DTWV}$. Representative diffraction limited mCherry signal and peptide-PAINT reconstructions for cells expressing vimentin fused to c-terminal mCherry or mCherry-pept$_{DTWV}$. Yellow line indicates the cellular outline. Both SMLM acquisitions were performed at 1 nM of PDZ-mNG in solution and reconstructions are displayed at identical scaling. Scale bar: 5µm.
Figure S7 (related to Figure 3). Characterization of binding kinetics of PDZ-mNG to pept_{DTWV} upon cellular expression and fixation. (a) Localizations over time for a representative peptide-PAINT acquisition in fixed cells expressing vimentin-mCherry-pept_{DTWV}, imaged at low PDZ-mNG concentrations and laser powers so that single binding events could clearly be distinguished. (b) Normalized frequency distribution of individual bright times (τ_b), for single binding events during a peptide-PAINT acquisition in fixed cells. Black line shows a one-phase exponential decay fit through the experimental data (bars, mean±sd, n=140327 single events).
Figure S8 (related to Figure 3). Preservation of the interaction between ePDZb1-mNG and pept_{DTWV} upon fixation allows for one-step staining in cells. Schematic representation of the constructs and interactions used in the experiments to detect and stain the pept_{DTWV} upon fixation with the high affinity ePDZb1 domain (left). Confocal images of the fixed mCherry expression marker for Vimentin and TOMM20 after expression and fixation in COS7. Corresponding confocal and STED images of ePDZb1-mNG to detect the constructs are depicted. Scale bar: 10µm (full cell); 2µm (zoom)
Figure S9 (related to Figure 4). Negative control for peptide-PAINT of vimentin-mCherry stained with \texttt{pept_{DTWV}} fused to \texttt{VHH_{mCherry}}. Diffraction limited mCherry signal and PDZ-mNG PAINT reconstruction for a cell expressing vimentin-mCherry, that was fixed and incubated with or without \texttt{VHH_{mCherry}}-\texttt{pept_{DTWV}}. Yellow line indicates the cellular outline. Both SMLM acquisitions were performed at 1 nM of PDZ-mNG in solution and reconstructions are displayed at identical scaling. Scale bar: 5 µm.
### Table S1. Overview of the peptide and protein domains used in this study.

| Construct                     | Amino Acid Sequence                                                                 | Fused to | linker  |
|-------------------------------|-------------------------------------------------------------------------------------|----------|---------|
| pet<sub>DTWV</sub> (last 4 amino acids are epitope for PDZ domains) | RGSIDTWV-cooh                                                                      | C-term   | GAG     |
| PDZ domain                    | M5MEIRVRVEKDPELGFSISGGVGRGNPPFPDPDDGIFVTRVPQEGPASKLQPGDKIIQOANQSGINIEHGQAVLKLTKFQOUNTRYLVEIVREVSS | N-term   | KL(HindIII restr site)-GSGS |
| ePDZ-b1 affinity clamp        | MPELGFSGSISGGVGRGNPPFPDPDDGIFVTRVPQEGPASKLQPGDKIIQOANQSGINIEHGQAVLKLTKFQOUNTRYLVEIVREVSS | N-term   | KL(HindIII restr site)-GSGS |
| VHH<sub>mcherry</sub>-pet<sub>DTWV</sub> | MGMHHHHHHMAQVQLVEGGSLVQPGGSLRLS CAASGRFAESSSSMGWFRQAPKEREFAVSWSG GATNYADSAGRTFLSRDNTKNTVLYQLMNSIKPD DTAYCAANLNLYSSQRLYWGQGTQTVS SPFTLEGAGRSIDTWV |          | GAG     |

### Table S2. Experimental conditions and imaging parameters.

| Figure sample                  | Probe            | Concentration | exposure time (ms) | # frames for reconstruction |
|-------------------------------|-------------------|---------------|--------------------|-----------------------------|
| Figure 1b (STED), Figure S2b  | nanoparticles     | ePDZb1-mNG    | 1 nM               | N/A                         | N/A                         |
| Figure 1b (SMMLM), Figure 1c  | nanoparticles     | PDZ-mNG       | 10 nM; Concentration was choosen until clear single molecule events could be observed at high laser intensity to optimize imaging for maximum amount of localizations with highest precision. Under these conditions, observing a constant number of single events is therefore likely a combination of continuous and bleaching and dissociation. | 60 | 23751 |
| Figure 2a-b, Figure S4a,b     | nanoparticles     | PDZ-mNG       | 26.9 nM            | 60                          | 30000                       |
| Figure 2c-d, Figure S4c-e     | nanoparticles     | PDZ-mNG       | 0.1 nM; In these experiments the probe concentration was choosen to observe full single binding events with a very low chance of overapping events. Acquisitions were performed at low laser powers where the mNG was not prone to bleaching before the probe dissociated. | 60 | 10000 |
| Figure 3a                      | cells             | PDZ-mNG       | Not determined; probe was diluted in blocking solution under the microscope until clear single molecule events could be observed at high laser intensity to optimize imaging for maximum amount of localizations with highest precision. | 50 | 30000 |
| Figure 3b                      | cells             | PDZ-mNG       | Not determined; probe was diluted in blocking solution until clear single molecule events could be observed at low laser intensity to optimize imaging for maximum amount of localizations with highest precision. | 60 | 26710 |
| Figure 4b                      | cells             | PDZ-mNG       | 1 nM               | 60                          | 30000                       |
| Figure 5a-b                    | nanoparticles     | PDZ-mNG       | 10 nM              | 60                          | 10000                       |
| Figure 5b                      | nanoparticles     | PDZ-mNG       | 10 nM              | 60                          | 10000                       |
| Figure 5c-d                    | nanoparticles     | PDZ-mNG       | 0.1 nM             | 60                          | 10000                       |
| Figure 5S                      | cells             | PDZ-mNG       | 1 nM               | 60                          | 20000                       |
| Figure 5S                      | cells             | PDZ-mNG       | 1 nM               | 60                          | 20000                       |
| Figure 5S                      | cells             | PDZ-mNG       | Not determined; probe was diluted in blocking solution until clear single molecule events could be observed at low laser intensity to avoid bleaching and be able to determine bright times of individual binding events. | 60 | 2000 |
| Figure 5S (STED)               | cells             | ePDZb1-mNG    | 50 nM              | N/A                         | N/A                         |
| Figure 5S (STED)               | cells             | ePDZb1-mNG    | 4 nM               | N/A                         | N/A                         |
| Figure S9                      | cells             | PDZ-mNG       | 1 nM               | 60                          | 20000                       |
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