A peptide tag-specific nanobody enables high-quality labeling for dSTORM imaging

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Dense fluorophore labeling without compromising the biological target is crucial for genuine super-resolution microscopy. Here we introduce a broadly applicable labeling strategy for fixed and living cells utilizing a short peptide tag-specific nanobody (BC2-tag/bivBC2-Nb). BC2-tagging of ectopically introduced or endogenous proteins does not interfere with the examined structures and bivBC2-Nb staining results in a close-grained fluorophore labeling with minimal linkage errors. This allowed us to perform high-quality dSTORM imaging of various targets in mammalian and yeast cells. We expect that this versatile strategy will render many more demanding cellular targets amenable to dSTORM imaging.
Fluorescence-based super-resolution microscopy (SRM) is becoming increasingly applied in cell biology. Single-molecule localization microscopy (SMLM) techniques, such as (direct) stochastic optical reconstruction microscopy ((d)STORM) provide outstanding spatial resolutions and have enabled unprecedented insights into the organization of subcellular components. However, the quality and value of SMLM imaging can be limited due to poor photon emission or detection efficiency, low fluorescence labeling densities, linkage errors or steric hindrances. Most current SMLM labeling approaches employ antibodies or recombinant proteins either fused to photoactivatable fluorescent proteins (FPs) or fluorogen-labeling enzymes, such as the Halo-, CLIP-, or SNAP-tag. While conventional antibodies introduce significant linkage errors by displacing the fluorophore from the target, large protein/enzyme tags can affect expression, cellular localization, folding and/or function of the respective fusion protein. Although small peptide tags, such as FLAG-, HA-, or Myc-tag are available, these epitopes often have to be arranged in multiple arrays to recruit medium-affinity binding antibodies and thus do not provide dense labeling sufficient for high-quality SRM.

Instead of using antibodies, a 15-amino-acid peptide-tag can be visualized by high-affinity fluorescently labeled monomeric streptavidin, which, however, can be affected by the binding of endogenously biotinylated proteins. Alternatively, reversibly on- and off-binding labels in point accumulation for imaging of nanoscale topography (PAINT) microscopy allow for a continuous and therefore ultra-high density readout as they are not limited by a predefined fluorophore tagging pattern. Yet, this approach can only be used for distinguishable structures like membranes or DNA combined with illumination-confined arrangements, such as in surface-near or lightsheet illuminations. The visualization of other structures by PAINT approaches relies on a specific labeling commonly achieved by DNA-Paint. As a promising substitute for conventional antibodies, small-sized nanobodies (antibody fragments derived from heavy-chain-only camelid antibodies) coupled to organic dyes were recently introduced for SRM. Nanobodies targeting native proteins, such as components of the nuclear pore complex, tubulin, or vimentin were described for dSTORM imaging. Despite their capability to directly probe endogenous antigens, the de novo generation of gene-specific nanobodies and their validation for SRM imaging purposes is cumbersome and time-consuming. Which is reflected by the fact that only a very limited number of SRM-compatible nanobodies are available by now. Due to their applicability for nanoscopy of widely used FP-fusions, GFP-, and RFP-nanobodies became very popular tools for SMLM imaging. However, this strategy relies on the correct expression of FP-fusions and does not cope with problems arising from mislocalization or dysfunction. Thus, nanobodies directed against short and inert tags might prove advantageous for SRM.

Here we introduce a versatile labeling and detection strategy comprised the short and inert BC2 peptide-tag (PDRKAAVSHWQQ) and a corresponding high-affinity bivalent nanobody (bivBC2-Nb) for high-quality dSTORM imaging. We demonstrate the benefits of our approach for close-grained fluorophore labeling with minimal linkage error of various ectopically introduced and endogenous targets in fixed and living cells.

Results
Development of a dSTORM suitable BC2-tag/bivBC2-Nb system. As originally described, we first labeled the BC2-Nb at accessible lysine residues by N-hydroxysuccinimide (NHS) ester fluorophores, such as Alexa Fluor 647 (AF647). While BC2-NbAF647 (NHS) is sufficient for wide-field microscopy (Fig. 1a, left panel, Supplementary Fig. 1a, b), dSTORM imaging of BC2-tagged proteins revealed a rather low-staining efficiency resulting in inferior structural labeling coverage (Fig. 1b, left panel). Thus, we analyzed the binding properties of a bivalent format of the BC2-Nb (bivBC2-Nb) (Fig. 1a, right panel). We assessed its binding kinetics by biolayer interferometry (BLI) and observed a considerably reduced dissociation rate compared to monovalent BC2-Nb (Supplementary Fig. 1c). Notably, this decrease in dissociation rate is not caused by simultaneous binding of the bivBC2-Nb to two BC2 epitopes as confirmed by a BLI assay using a tandem-BC2-tag of two consecutively linked BC2 epitopes (BC2-BC2-tag) (Supplementary Fig. 1d).

Nevertheless, antigen labeling using the bivBC2-Nb conjugated to NHS-ester fluorophores did not yield the expected visual improvement of staining specificity (Fig. 1a, right panel). Considering the crystal structure, we designed a site-directed, enzymatic coupling strategy, which should not affect the paratope and binding properties of bivBC2-Nb. Using the Sortase-A system, we linked peptides conjugated to a single-AF647 fluorophore in a defined 1:1 ratio to the C-terminus of bivBC2-Nb (bivBC2-NbAF647 (sort)) (Fig. 1a, right panel, Fig. 1c and Supplementary Fig. 1a, b, c). An exemplary dSTORM image of a HeLa cell transiently expressing vimentin (Fig. 1d). However, this level is considerably lower compared to bivBC2-NbAF647 (NHS) (Fig. 1a, right panel, Supplementary Fig. 1a, b, c). An exemplary dSTORM image of a HeLa cell transiently expressing vimentin (Fig. 1d).

For a better understanding bivBC2-NbAF647 (sort) is referred to bivBC2-NbAF647 in the following.

Since the BC2-Nb was originally developed against β-catenin, we assessed the influence of the background staining of endogenous β-catenin on the labeling quality. To distinguish background due to general unspecific staining from additional β-catenin staining, we compared HeLa cells (not expressing any GFP epitope) stained with a GFP-targeting nanobody (GFP-NbAF647) to HeLa cells stained with bivBC2-NbAF647. Further, we performed bivBC2-NbAF647 staining in HeLa cells transiently expressing the non-structural, autophagosomal marker protein LC3B fused to the BC2-tag (bivBC2-NbAF647). This is - in the absence of autophagy - homogeneously distributed throughout the cytoplasm. By analyzing the dSTORM data using DBSCAN clustering, we measured a slightly increased level of 1.7 (±0.3 S.D.) nanobodies per square micrometer for bivBC2-NbAF647 compared to the unspecific background staining of 0.61 (±0.03 S.D.) GFP-NbAF647 per µm². However, this level is considerably lower compared to 7.2 (±1.3 S.D.) bivBC2-NbAF647 per µm² which we observed for the staining of BC2-LC3B expressing cells (Fig. 1d, Supplementary Fig. 2a). We then compared signal intensities derived from bivBC2-NbAF647-stained HeLa cells, which were either left untreated or incubated with CHIR99021 (CHIR) to accumulate endogenous β-catenin. While immunolabeling with a β-catenin-specific antibody showed a strong enrichment in CHIR-treated cells (Supplementary Fig. 2b), dSTORM imaging revealed only a minor increase of bivBC2-NbAF647 localizations (Supplementary Fig. 2c, left panel). Moreover, in CHIR-treated HeLa cells transiently expressing vimentin (Fig. 2d).

From this we conclude that even if present at high...
levels, the BC2-epitope of β-catenin has a negligible impact on staining of ectopically introduced antigens.

For a stoichiometric quantification of the labeling quality of the BC2-tag/bivBC2-Nb detection system, we utilized the *Escherichia coli* protein ferritin (FtnA) recently described as a homooligomeric protein standard of 24 subunits[36]. We expressed BC2-tagged, as well as SNAP-tagged FtnA-24mers in U2OS cells and performed dSTORM imaging on cell lysates immobilized on coverslips[36]. By measuring single-AF647 blinking events, we obtained the parameters of the corresponding log-normal distribution ($\mu = 5.68$, $\sigma = 0.4$), which describes the probability distribution of single-molecule fluorescence intensities (Supplementary Fig. 3a). We then measured the fluorescence intensities of immobilized FtnA oligomers labeled with the BC2-tag/bivBC2-Nb or SNAP-tag system. We compared these distributions to the expected fluorescence intensity distributions of fully labeled FtnA-24mers, calculated from the single-molecule fluorescence intensity distribution and the degree of labeling of each component (Methods section). As a result, the BC2-tag/bivBC2-Nb FtnA-oligomer staining revealed a completeness of labeling of
61.4% which competes with the covalent SNAP-tag FtnA-staining efficiency of 64.7% (Fig. 1e, Supplementary Fig. 3b) and outcompetes photoactivation/photoconversion efficiencies of fluorescent proteins.\(^{37}\) Notably, our observation of a rather low efficiency of about 65% for the SNAP-tag labeling is in agreement with reported assessments\(^{36,38}\), and conference presentations by K. Yserentant (2017).

**Comparison of different labeling strategies for SMLM.** As genetic tagging of structural proteins like vimentin often impairs their structure and function\(^{34,40}\), we evaluated the influence of the short BC2-tag on vimentin structure formation and compared the BC2-tag/bivBC2-Nb detection system with established strategies focusing on image quality and apparent organization of the vimentin network. We performed SMLM on native vimentin in comparison to vimentin fused to photoactivatable mCherry (PAmCherry-vimentin), eGFP (GFP-vimentin), or the BC2-tag (Fig. 2a, b). For our studies, we transiently expressed mCherry (PAmCherry-vimentin), eGFP (GFP-vimentin), or the vimentin in comparison to vimentin fused to photoactivatable the short BC2-tag on vimentin structure formation and com-
fitted vimentin network (Supplementary Fig. 5). Obviously, the corresponding proteins for 24 h in HeLa cells followed by chemical fixation of the cells. Native vimentin was visualized with the recently described vimentin-specific nanobody bivVB6-Nb\(_{AF647}\)^{25,41}, while PAmCherry-vimentin was mapped directly. The other constructs were labeled with the nanobodies GFP-Nb\(_{AF647}\) or bivBC2-Nb\(_{AF647}\), respectively. Image analysis of the vimentin network visualized by the different labeling strategies revealed considerable phenotypic differences (Fig. 2b, c; images of all cells quantitatively analyzed Supplementary Fig. 4a–d; analysis routine Supplementary Fig. 4e and Methods section). 94% of native vimentin fibers labeled with the bivVB6-Nb\(_{AF647}\) showed widths below 150 nm. In contrast, cells with incorporated PAmCherry-vimentin were smaller and showed a high percentage (20%) of thick vimentin bundles above 150 nm width, while in GFP-vimentin expressing cells more than 96% of all detectable vimentin fibers had widths below 75 nm (Fig. 2c). Compared to the N-terminally labeled counterparts, cells expressing vimentin-PAmCherry displayed a highly similar phenotype whereas cells expressing vimentin C-terminally fused to GFP (vimentin-GFP) showed an even more severely frag-
mented vimentin network (Supplementary Fig. 5). Obviously, both type and position of the FP affects the formation of the vimentin network and induce altered cellular phenotypes. The various observed morphological alterations are likely caused by several mislocalization and self-oligomerization artifacts induced by the different FP moieties derived either from jelly fish (GFP) or red corals (DsRed)\(^{40,42}\). Notably, no phenotypic changes or significant differences in the abundance of fiber widths were detected between native and BC2-tagged vimentin (96% of all fibers below 150 nm, 4% above 150 nm; Fig. 2b, c and Methods section).

We then assessed the SMLM image quality achievable by the different labeling approaches. The quality is dependent on two main factors; (i) the optical resolution dictated by the precision with which fluorescent spots can be localized, and (ii) the structural resolution determined by the labeling density (coverage) and the physical distance between fluorophore and target (linkage error). We assessed these parameters for each analyzed fiber individually. The localization precision was calculated by a Nearest Neighbor based Analysis (NeNA)\(^{43}\), the labeling density was determined by the lengthwise fluorescent signal coverage along each fiber, and the linkage error by quantifying the apparent width of fibers of the smallest fiber category. For further comparison, we calculated the Fourier Image RESolution (FIRE) values\(^{44}\) (see Supplementary Note 1). Since the readout of all three nanobody labeling strategies relies on the same bright fluorophore (AF647), NeNA yielded the same optical resolution statistics with a mean NeNA localization precision of about 9–12 nm. The fluorescent-protein PAmCherry has a lower photon yield and achieves an average NeNA value of 17 nm (Supplementary Fig. 6). The structural resolution as assessed by the different labeling coverage statistics revealed significant differences (Fig. 2c, Supplementary Fig. 6 and Methods section). For PAmCherry-vimentin, we observed the lowest coverage among all labeling strategies for thin fibers, and a maximum coverage of ~75% for thick fibers, which is likely due to inefficient chromophore formation and photoactivation. The low coverage of ~50% for the GFP-Nb is more likely explained by a steric hindrance in incorporating GFP-tagged molecules into the native vimentin network, which is in line with our observation of only thin fibers. The highest labeling coverage was observed for bivBC2-Nb with a coverage of ~80% for fibers below 75 nm width, and nearly full coverage of fibers exceeding a width of 150 nm. For thin fibers it exceeds the coverage obtained with the bivVB6-Nb for native vimentin, which might be due to a reduced accessibility of the native epitope within assemblen vimentin filaments. To assess the impact of the size of the labeling probe on the structural resolution, we compared our bivBC2-Nb-based approach with conventional, monoclonal antibody staining (Supplementary Fig. 7a). Antibody labeling resulted in nearly complete coverage of thin vimentin fibers (>75 nm) (Supplementary Fig. 7b), and the AF647-based readout resulted in the same localization precision and optical

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**Fig. 1** Comparison and characterization of BC2-nanobody (BC2-Nb) formats for wide-field and dSTORM imaging. a Schematic illustration of the BC2-Nb dye-conjugation strategies. Monovalent and bivalent BC2-Nbs were either conjugated with Alexa Fluor 647 (AF647) via N-hydroxysuccinimide (NHS) ester (left panel) or linked to AF647 by enzymatic sortase coupling (right panel). Wide-field imaging of chemically fixed HeLa cells expressing mCherry-vimentin\(_{NC2}\) (mCherry-VM\(_{BC2}\)) stained with modified BC2-Nbs. Monovalent versions of the BC2-Nbs (NHS- and sortase-coupled) are depicted on the left panel, corresponding bivBC2-Nbs are displayed on the right side. Stainings with NHS-conjugated nanobodies are shown in two different image contrasts, the upper half in the same brightness and contrast as the sortase-coupled nanobodies; in the lower half with an adjusted contrast. Scale bars, 25 \(\mu\)m. b Representative dSTORM images of chemically fixed HeLa cells expressing vimentin\(_{BC2}\), stained with the monomeric NHS-conjugated BC2-Nb\(_{AF647}\) (NHS) (left) and the sortase-coupled bivBC2-Nb\(_{AF647}\) (sort) (right). Scale bars, images 5 \(\mu\)m, insets 1 \(\mu\)m. Image reconstruction details are given in Methods section. c Assessment of staining quality in wide-field fluorescence imaging. Labeling of the different nanobody formats was quantified by calculating the ratio of the signal intensity of mCherry-VM\(_{BC2}\) expressing cells to non-transfected cells (background), (BC2-Nb\(_{AF647}\) NHS): \(n = 134\); BC2-Nb\(_{AF647}\) (sort): \(n = 150\); bivBC2-Nb\(_{AF647}\) (sort): \(n = 195\) (Methods section, Supplementary Fig. 1). d Assessment of bivBC2-Nb\(_{AF647}\) staining of endogenous \(\beta\)-catenin. Bar chart summarizes measured nanobody per \(\mu\)m\(^2\) values for untransfected chemically fixed HeLa cells stained with GFP-\(\text{N}^{\text{GFP}}\) or bivBC2-Nb\(_{AF647}\) in comparison to chemically fixed HeLa cells transiently expressing BC2TLC3B stained with bivBC2-Nb\(_{AF647}\), errors given as standard deviation (S.D.), \(N = 3\) cells for each condition (Methods section, Supplementary Fig. 2). e Quantification of completeness of labeling for the bivBC2-Nb and SNAP-tag labeling systems using FtnA-oligomers of 24 subunits. Bar chart summarizes median values of FtnA-24mer fluorescence intensities as percentage of theoretical maxima (Methods section, Supplementary Fig. 3).
resolution statistics (Supplementary Fig. 7c). Despite the high-labeling coverage, the antibody-mediated displacement of the fluorophore led to an increased linkage error. Accordingly, we measured an average width of ~55 nm for thin vimentin fibers probed with the antibody, whereas a smaller apparent width of ~40 nm was observed with bivBC2-Nb (Supplementary Fig. 7c).

Detection of various cellular targets with the bivBC2-Nb. Next we analyzed whether the BC2-tag/bivBC2-Nb detection system is transferable to other structural proteins. To test whether orientation of the BC2-tag affects the incorporation of the recombinant protein into endogenous structures, we transiently expressed cDNAs of mouse TUBA1B, human LMNB1, or ACTB either comprising the BC2-tag on the N- or the C-terminus in different cell lines followed by detection with bivBC2-Nb. As exemplarily shown for tubulin alpha-1B, C-terminal addition of the BC2-tag yielded more distinct microtubule structures compared to the N-terminally tagged version (Supplementary Fig. 8). For lamin B1, we observed no differences regarding the tag position whereas ectopically expressed β-actin is only incorporated into the actin cytoskeleton when the BC2-tag is located at the N-terminus, which is in accordance to previously tested tagging approaches. dSTORM imaging of HeLa cells expressing either laminBC2T or BC2Tactin, as well as U2OS cells transiently expressing tubulinBC2T revealed that BC2-tagged proteins are efficiently incorporated in the corresponding structures and could be imaged at high-resolution reaching localization precisions of 9–12 nm as previously shown for vimentinBC2T (Fig. 3a–c, Supplementary Fig. 9). As individual microtubules have a defined diameter of 25 nm these structures serve as an experimental benchmark for SRM. Simulations on nanobody labeling of microtubules using a maximal probe displacement of 5 nm and a localization precision cutoff of 10 nm have yielded an apparent fiber width of about 40 nm, which is in perfect agreement to our measured fiber width of 38.2 ± 9.2 nm (Supplementary Fig. 9a). Moreover, a detailed analysis of individual actin fibers comprising transiently expressed BC2Tag actin showed comparable labeling densities as previously obtained for vimentinBC2T (Supplementary Fig. 9b–d).

Additionally, we used our approach to visualize non-structural proteins, namely the autophagosomal marker protein LC3B and the extracellular membrane marker GFP-GPI. To monitor induction of autophagy, we co-expressed BC2T-LC3B and GFP-LC3B in HeLa or A549 cells followed by incubation with DMSO or rapamycin to induce autophagosome formation. Wide-field imaging of chemically fixed cells, stained with bivBC2-NbARose, showed a clear co-localization of GFP and nanobody signals at defined spots in rapamycin-treated cells, indicating correct localization of BC2-tagged LC3B at autophagosomes.
Further, dSTORM imaging and subsequent DBSCAN cluster analysis of newly formed autophagosomes in BC2T-LC3B expressing cells after incubation with rapamycin revealed diameters of ~0.3–1.0 µm for these foci (Fig. 3d, Supplementary Fig. 11, Methods section), which is in accordance to previous findings\(^50, 51\). For BC2-tagged GFP-GPI (BC2TGFP-GPI), we observed a clear co-localization of the nanobody and the GFP signal at the plasma membrane in chemically fixed HeLa cells (Supplementary Fig.10b). Notably, with dSTORM, we detected a defined spatial organization of BC2TGFP-GPI, e.g., the formation of small clusters and enrichment of BC2TGFP-GPI molecules at cell–cell contacts, compared to a diffraction-limited, homogenous distribution observable by wide-field microscopy (Fig. 3e, Supplementary Fig.10b).

Visualization of endogenous proteins with the bivBC2-Nb. To utilize the BC2-tag as an endogenous marker under native promoter expression, we first replaced the gene coding for the nuclear DNA-binding protein cbp1 at its endogenous loci in the fission yeast Schizosaccharomyces pombe (S. pombe) by a C-terminally BC2-tagged version (cbp1\(_{BC2T}\)). Cells expressing cbp1\(_{BC2T}\) show growth rates comparable to wild type (wt) and exhibit no morphological changes (Supplementary Fig.12a, b). As S. pombe possesses a thick cell wall and a highly packed cellular environment, any immunofluorescence-based SRM approach suffers from high-unspecific background and low-staining quality. Notably, by staining endogenously expressed cbp1\(_{BC2T}\) utilizing the bivBC2-NbAF647 we were now able to visualize an endogenous nuclear protein in S. pombe by dSTORM imaging (Supplementary Fig. 12a). Second, we stably introduced the coding sequence of the BC2-tag under the native \(\beta\)-actin promoter at the 5' end of the first exon of endogenous \(\beta\)-actin in HeLa and A549 cells using the CRISPR/Cas9 technology. After monoclonal selection of cells exhibiting a heterozygous integration of BC2T\(_{\beta\text{-actin}}\) (HeLa\(_{\beta\text{-actin}}\); A549\(_{\beta\text{-actin}}\), Methods section) we treated both cell lines with transforming growth

Fig. 3 Super-resolution imaging of transiently expressed BC2-tagged proteins in chemically fixed cells. Representative dSTORM images of a a HeLa cell expressing BC2T\(_{\text{lamin}}\), b U2OS cell expressing tubulin\(_{BC2T}\) (filament width statistics in Supplementary Fig. 9a), c HeLa cell expressing BC2T\(_{\text{actin}}\) (coverage statistics in Supplementary Fig. 9b), d HeLa cells expressing BC2T-LC3B either left untreated or treated with rapamycin. Bar charts represent the degree of clustering, given as a relative fraction of cluster points versus noise points, errors given as standard deviation (S.D.). Histograms represent cluster diameters as determined by DBSCAN analysis with a bin size of 100 nm (x-axis) plotted against relative fraction (y-axis). Full data are represented underneath the histograms as box diagram plots with the same x-axis. The box marks the three quartiles and the whiskers mark 95% of all the data. Total number of clusters \(n = 342\) in non-treated cells, \(n = 405\) in treated cells, \(N = 3\) cells for untreated cells and \(N = 4\) cells for rapamycin-treated cells (Supplementary Fig. 11), and e HeLa cells expressing BC2T-GFP-GPI. All cells were stained with bivBC2-NbAF647 (Methods section). Scale bars, images 5 µm, insets 1 µm. Crossed out rectangles mark the position of fiducial markers used for drift correction. Image reconstruction details are given in Methods section.
factor β (TGFβ) and monitored the induction of actin stress fibers by co-staining with bivBC2-Nb<sub>AF647</sub> and phalloidin<sub>AF555</sub>. As expected, we detected the formation of stress fibers only in A549 wt and A549-BC2TACTB cells, which are described to respond to TGFβ<sub>52</sub> (Fig. 4a). dSTORM imaging further allowed a detailed insight into the non-disturbed actin network (Fig. 4b). These findings indicate that BC2-tagging of endogenous proteins is a viable approach for SRM studies to visualize cellular targets at endogenous levels and with minimal functional interference.

**bivBC2-Nb visualizes its target proteins in living cells.** To realize the advantages of the BC2-tag/bivBC2-Nb system also for live-cell applications, we first performed time-lapse imaging of HeLa cells transiently expressing BC2-GFP-GPI. After addition of bivBC2-Nb<sub>AF647</sub> to the imaging medium, we observed a fast recruitment of the nanobody to its membrane-located antigen (Supplementary Fig. 13a), with a saturation of the nanobody signal within 20–30 min (Supplementary Fig. 13b). Single-particle tracking dSTORM imaging further allowed us to trace the highly dynamic behavior of the nanobody in living cells.

**Fig. 4** Visualization of endogenously expressed BC2-tagged actin labeled with bivBC2-Nb<sub>AF647</sub>. **a** Wide-field images of chemically fixed wild-type A549 and HeLa (-wt; left panel), as well as chemically fixed A549-BC2TACTB and HeLa-BC2TACTB cells (right panel). Cells were either left untreated (0 h) or stimulated for 48 h with TGFβ (5 ng ml<sup>−1</sup>) followed by staining with phalloidin<sub>AF555</sub> and bivBC2-Nb<sub>AF647</sub>. Scale bars, 25 µm. **b** dSTORM image of a representative HeLa-BC2TACTB cell. Scale bars, image 5 µm, insets 1 µm. Image reconstruction details are given in Methods section. Imaging sequence taken from raw data acquisition can be found in Supplementary Movie 5, assessment of AF647 photophysics under dSTORM imaging conditions can be found in Supplementary Fig. 15.
Discussion

In this study, we developed and extensively characterized a broadly applicable and transferable labeling strategy based on a structurally minimal tag in combination with the first peptide-specific nanobody suitable for SRM. In contrast to the widely established GFP/GFP-Nb system\(^1\),\(^2\) for dSTORM imaging of fusion proteins comprising a large fluorescent moiety, the short and inert BC2-tag allows an efficient and dense incorporation of ectopically and endogenously expressed proteins into higher-ordered cellular structures in mammalian and yeast cells. In particular, it does not interfere with the native organization of structural proteins, such as vimentin, lamin, actin, and tubulin.

Dynamic movements of thousands of BC2\_GFP-GPI molecules along the plasma membrane in high spatial and temporal resolution, e.g., the increased dynamics at cell-to-cell contact areas (Fig. 5a, Supplementary Movies 1–4). Second, for bivBC2-Nb staining of intracellular targets in living cells, we adapted a lipid-based protein transfection protocol\(^5\),\(^3\),\(^4\) and introduced bivBC2-Nb conjugated to different dyes into our cell lines HeLa-BC2TACTB and A549-BC2TACTB. Within 2 h, we observed cellular uptake of nanobody and its accumulation at the actin cytoskeleton irrespective of the attached fluorophore (Supplementary Fig. 14a). Prolonged time-lapse imaging of live HeLa-BC2TACTB cells for 5 h revealed a stable staining of the cellular actin network of HeLa-BC2TACTB cells (Fig. 5b) with sub-diffraction details as previously seen for chemically fixed HeLa-BC2TACTB cells (Fig. 4b). We further documented the photophysical differences in performance of the ATTO655 fluorophore, which is outcompeted by AF647 both in blinking statistics and brightness (Supplementary Fig. 15, Supplementary Movies 5 and 6). In summary, these data demonstrate that the bivBC2-Nb is functional within living cells where it retains its outstanding binding capacities allowing for live-cell dSTORM imaging. Other recently reported protein transduction approaches offer numerous alternatives to introduce the bivBC2-Nb in various cell types for live-cell imaging of BC2-tagged proteins\(^3\),\(^5\)–\(^6\).

Fig. 5 Super-resolution imaging and single-particle tracking in live HeLa cells. a dSTORM image of live BC2\_GFP-GPI expressing HeLa cells stained with bivBC2-Nb\_AF647 and insets in gray scale overlaid with single-particle trajectories of immobile (diffusion coefficient below 0.02 \(\mu m^2 s^{-1}\), left) and mobile (diffusion coefficient above 0.02 \(\mu m^2 s^{-1}\), right). Color scale of diffusion coefficients is given under insets. Scale bars 10 \(\mu m\) in images, 2 \(\mu m\) in insets. Supplementary Movies 1–4 show the recorded live BC2\_GFP-GPI dynamics of the whole cell and the corresponding insets. b Live-cell dSTORM images of two HeLa-BC2TACTB cells stained with bivBC2-Nb\_ATTO655. Wide-field fluorescence images in upper left corners. Scale bars in 10 \(\mu m\), 2 \(\mu m\) in insets. Image reconstruction details are given in Methods section. Imaging sequence taken from raw data acquisition can be found in Supplementary Movie 6, assessment of ATTO655 photophysics under live cell dSTORM imaging conditions can be found in Supplementary Fig. 15.
**Method**

**Expression constructs.** All primer sequences used for cloning are listed in a Supplementary Table 1. The expression construction coding for vimentin N-terminally fused to eGFP (GFP-vimentin) was previously described. For generation of a photoactivatable (PA) mCherry (PamCherry) fusion construct of vimentin (PamCherry-vimentin) the coding sequence of PamCherry was PCR-amplified from the pBAD/HisU-PamCherry vector using the following primer set: PamCherry-F and PamCherry-R. The PCR product was purified with the restriction enzymes Agel and BgIII and ligated in the Agel/BgIII sites of a vector coding for mCherry-vimentin thereby replacing mCherry with PamCherry. An expression construct coding for vimentin with a C-terminal BC2-tag (vimentinBC2T) was generated by the replacement of the mCherry sequence from a mCherry-VimentinBC2T (mCherry-vimentinBC2T) fusion construct previously described. Thus, vimentinBC2T cDNA was PCR-amplified using the primer VMBC2T-for and VMBC2T-rev. Both DNA fragments were purified and ligated into Nhel and BamHI restriction sites of the template construct. Constructs coding for vimentin C-terminally fused to PamCherry (vimentin-PamCherry) and vimentin C-terminally fused to eGFP (vimentin-GFP) were generated by Gibson assembly of the three following fragments: fragment 1 - pEGFP-N1 vector backbone digested with Nhel and BsrGI fragment 2 - vimentin amplified from vimentinBC2T with the primer set VM-for and VM-rev, fragment 3 - PamCherry amplified from PamCherry-vimentin or eGFP amplified from GFP-vimentin using the primer set PamCherry/eGFP-for and PamCherry/eGFP-rev. Fragments were assembled using the Gibson-Assembly Master mix (New England Biolabs, cat. #E2611) according to the manufacturer’s protocol. An expression construction coding for BC2-tagged β-actin (BC2T-actin) was generated by the combination of two PCR fragments derived from an eGFP-actin construct previously described in ref. Thus, the first PCR fragment was generated using primer B2T-actin(2f)-rev and B2T-actin(2r)-for. The second PCR fragment was generated using primer BC2TActb(2)-for and BC2TActb(2)-rev. Both DNA fragments were purified and ligated by compatible sticky ends generated by BssHII and SseA1 restriction enzymes. To generate a BC2-tagged laminB1 (laminBC2T) expression construct the lamin B1 cDNA was PCR-amplified from a GFP-Lamin B1 DNA template using PCR primers B2TLaminBC2T-for and B2TLaminBC2T-rev and cloned into XhoI and NheI restriction sites of pEGFP-N1 vector backbone digested with NheI and BsrGI, fragment 2 - vimentinCas10A (vimentin-PAmCherry) and vimentin C-terminally fused to eGFP (vimentin-GFP) amplified from pEGFP-C1 vector backbone digested with BsrGI and XhoI restriction sites. The resulting expression constructs were confirmed by sequencing and SDS-PAGE followed by blue stain analysis using antibodies directed against eGFP (ChromoTek, cat. #3H9, dilution 1:1000), mCherry (ChromoTek, cat. #25, dilution 1:4000) or a BC2-Nb coupled to Alexa Fluor 647 (Thermo Fisher Scientific, cat. #A20017). For all three constructs the original tag was replaced by a Sortase-tag (stated for cat. #A20017). For BC2-Nb and bivBC2-Nb NHS-conjugated nanobodies DOAs of 1.8 ± 0.5 and 2.1 ± 0.7 were determined.

**Bio-layer interferometry (BLI).** The dissociation constants of BC2-Nb and bivBC2-Nb were determined on BLItz system (Pall ForteBio). Synthetic BC2 and BC2-BG (with (GGGGS)n linker) peptides with an N-terminal biotin-Doa-Linker (Doa = 5-amino-1-carboxy-2-pyrrolidinone) were immobilized on streptavidin-coated biosensors (Pall ForteBio, cat. #18-5020) using a concentration of 50 nM. For kinetic measurements of BC2- or bivBC2-Nbs three concentrations (120 nM, 240 nM, and 480 nM) of the Nbs in diluent buffer (1× PBS, 0.1% (w/V) BSA) were used. Each measurement was done in duplicates with an association time of 180 s followed by 240 s dissociation in diluent buffer. Kinetic constants were determined using BLItz software (BLItz Pro 1.2, Pall ForteBio) according to global fitting of data sets.

**Cell culture and transfection.** The HeLa Kyoto cell line (Cellosaurus no. CVCL_1922) was obtained from S. Narumiya (Kyoto University, Japan), and the A549, U2OS and COS-7 cell lines were obtained from ATCC (CCL-185, HTB-96, CCL-132). All cell lines were used at passages negative for mycoplasma using the PCR mycoplasma kit Venor GeM Classic (Minerva Biolabs, cat. #11-1025) and the Taq DNA Polymerase (Minerva Biolabs, cat. #53-1010). Since this study does not include cell line specific analysis, all cell lines were used without additional authentication. HeLa Kyoto, U2OS and COS-7 cells were cultured in DMEM/10% Trypsin 0.05% (Pall ForteBio, cat. #18-1004) supplemented with 10% FCS (Life Technologies, cat. #10270-106), 1 unit ml−1 pen/strep (Life Technologies, cat. #15140-122). A549 cells were cultured in DMEM/F-12 (1:1) (Life Technologies, cat. #21331-020) supplemented with 10% FCS (Life Technologies, cat. #10270-106) and 1 unit ml−1 pen/strep (Life Technologies, cat. #15140-122). A549 cells were cultured in DMEM/F-12 (1:1) (Life Technologies, cat. #21331-020) supplemented with 10% FCS (Life Technologies, cat. #10270-106), 1 unit ml−1 pen/strep (Life Technologies, cat. #15140-122) and 2 mM l-glutamine (Life Technologies, cat. #25300-024). Cells were trypsinized for passage and cultivated at 37 °C in a humidified chamber with a 5% CO2 atmosphere. Transient transfection of HeLa Kyoto, U2OS, and COS-7 cells with Lipofectamine 2000 (ThermoFishier Scientific, cat. #11680-019) and transfection of A549 cells with Lipofectamine LTX (ThermoFisher Scientific, cat. #15338810) was performed according to the manufacturer instructions.

S. pombe strain construction. The cloning strategy for BC2-tagging of the CBP1 gene at the C-termius was adapted from ref. The Saccharomyces cerevisiae ADH1 terminator and kanamycin resistance gene were amplified from the PAWS plasmid using the following primer pair F_KanR_BC2 and R_KanR. ~250 bp sequences up- and downstream of the cbp1 gene were amplified from purified S. pombe DNA with the primer pairs F1_chp1, chp2 BC2-R1, and F2_chp1, chp2. Primers were designed to generate PCR products with overlapping regions of at least 20 bp. DNA fragments were assembled with overlap-extension PCR, using melting temperatures of the overlapping regions as the annealing temperature. All PCRs were performed with Q5 High-Fidelity DNA polymerase (New England Biolabs, cat. #M0491L). Volume of 10 µl of the PCR product was transformed into wild-type S.pombe using the Frozen-Easy Yeast Transformation II Kit (Zymo Research, cat. #ZT2001), plated onto YES agar plates and incubated overnight at 30 °C, then replica plated onto 200 µg ml−1 G418 (Thermo Fisher Scientific, cat. #R930-25, dilution 1:1000). An expression vector coding for SortaseA59 (pET28a-StAdelta59) was a gift from Hidde Ploegh (Addgene plasmid #51138).

**Recombinant protein production and nanobody labelling.** GFP-Nb, bivVB6-Nb, BC2-Nb, and bivBC2-Nb all comprising a C-terminal Sortase-tag were expressed and purified as previously described, and stored at ~80 °C or immediately used for labeling. SortaseA59 was expressed and purified as described. Alexa Fluor 647 (AF647)-coupled peptide H-Gly-Gly-Gly-Doa-Lys-NH2 (sortase substrate) was purchased from Intavia AG. Chemical dye conjugation of BC2-Nb or bivBC2-Nb was carried out as described previously. Briefly, purified nanobody was labeled with the N-hydroxysuccinimide (NHS) ester activated AF647 (ThermoFisher Scientific, cat. #A20006) according to manufacturer’s guidelines. After coupling, unbound dye was removed by separation on Zea Spin Desalting Columns (ThermoFisher Scientific, cat. #A20006). For analysis of the SortaseA59, A59 was subjected to SDS-PAGE and analyzed on a Typhoon Trio (GE-Healthcare, excitation 633 nm, emission filter settings 670 nm BP 30) and subsequent Coomassie staining. Degree of labeling (DOL, dye-to-protein ratio) was determined by absorption spectroscopy according to the instructions provided by ThermoFisher Scientific (stated for cat. #A20017). For BC2-Nb and bivBC2-Nb NHS-conjugated nanobodies DOAs of 1.8 ± 0.5 and 2.1 ± 0.7 were determined.

**Presentation of the results.** The results were presented as mean ± standard deviation, and statistical analysis was performed by one-way ANOVA followed by Tukey’s post hoc test or by Mann-Whitney U test using Prism7 software (GraphPad). All statistical tests were performed at a significance level of P<0.05.
S. pombe staining. ACTB target gene locus using the primer ACTB_fw and BC2_rev. PCR products

were designed for the previously cleaned with a 2% solution of Hellmanex III (Helma Analytics).

60) target gene locus using an online CRISPR gRNA design tool68, 69 and syn-

4% PFA (Sigma-Aldrich, F8775) and 0.25% (w/V) glutaraldehyde (Sigma-Aldrich, 

#G5882) in PEM for 10 min, then washed 2x with PEM and immobilized on pol-

yl-lysine coated Ibidi 8-well glass bottom slides (Ibidi GmbH, cat. #80826), previously cleaned with a 2% solution of Hellmanex III (Helma Analytics).

CRISPR/Cas9/D10A expression vector construct and HDR template. Paired sgRNAs were designed for the ACTB (actin beta, Homo sapiens; PubMed Gene ID: 60) target gene locus using an online CRISPR gRNA design tool68, 69 and syn-

thesized as Ultramer DNA Oligonucleotides (IDT). HDR

ACTB_hdr was synthesized as Ultramer DNA Oligonucleotides (IDT). HDR

template for the ACTB target gene locus.

Generation of BC2-tag knock-in cell lines. 1 x 10^6 HeLa Kyoto and A549 cells, respectively, were co-transfected at 50% confluency with 6.5 µg ACTB HDR template oligonucleotide and 5.5 µg cloned Cas9/Puro_ACTB_sgrna expression vector construct into a 6 well plate (to transfection efficiency) respectively. For transfection of hela cells Lipofectamine RecBcd (New England Biolabs, cat. #M0345L) the column purified DNA plasmid was digested overnight at 37 °C with BbsI. The linearized plasmid was then ligated into the BbsI-digested pDonor U6 plasmid (a gift from Andrea Ventura, Addgene plasmid #69312)87 using the NEBuilder Cloning Kit (New England Biolabs, cat. #E5520S). After treatment with Exonuclease RecBCD (New England Biolabs, cat. #M0345L) the column purified DNA plasmid was digested over night at 37 °C with BbsI. The linearized plasmid was then ligated into the BbsI-digested and dephosphorylated pSpCas9n(BB)-2A-Puro plasmid (a gift from Feng Zhang, Addgene plasmid #62987)50 using T4 DNA ligase (NEB). Single-cloned derived DNA plasmids were purified using QIAGEN Plasmid Midi Kit (Qiagen, cat. #12145) and verified by sequencing. The homology directed repair (HDR) template ACTB_HDR was synthesized as Ultramer DNA Oligonucleotides (IDT). HDR templates encoded for the HDR insert carrying the intended BC2-tag knock-in mutation flanked by left and right homology arms (each 50 bp) homologous to the ACTB target gene locus.

Generation of BC2-tag knock-in cell lines. 1 x 10^6 HeLa Kyoto and A549 cells, respectively, were co-transfected at 50% confluency with 6.5 µg ACTB HDR template oligonucleotide and 5.5 µg cloned Cas9/Puro_ACTB_sgrna expression vector construct into a 6 well plate (to transfection efficiency) respectively. For transfection of hela cells Lipofectamine RecBcd (New England Biolabs, cat. #M0345L) the column purified DNA plasmid was digested over night at 37 °C with BbsI. The linearized plasmid was then ligated into the BbsI-digested and dephosphorylated pSpCas9n(BB)-2A-Puro plasmid (a gift from Feng Zhang, Addgene plasmid #69312)87 using the NEBuilder Cloning Kit (New England Biolabs, cat. #E5520S). After treatment with Exonuclease RecBCD (New England Biolabs, cat. #M0345L) the column purified DNA plasmid was digested over night at 37 °C with BbsI. The linearized plasmid was then ligated into the BbsI-digested and dephosphorylated pSpCas9n(BB)-2A-Puro plasmid (a gift from Feng Zhang, Addgene plasmid #62987)50 using T4 DNA ligase (NEB). Single-cloned derived DNA plasmids were purified using QIAGEN Plasmid Midi Kit (Qiagen, cat. #12145) and verified by sequencing. The homology directed repair (HDR) template ACTB_HDR was synthesized as Ultramer DNA Oligonucleotides (IDT). HDR templates encoded for the HDR insert carrying the intended BC2-tag knock-in mutation flanked by left and right homology arms (each 50 bp) homologous to the ACTB target gene locus.

Immunoﬂuorescence staining for wide-field microscopy. For immuno-

fluorescence staining ~1.5 × 10^4 HeLa Kyoto, U2OS cells, COS-7, or A549 cells per well of a µclear 96-well plate (Greiner Bio One, cat. #655900) and cultured at standard conditions. Next day, Nbs were transfected using Pro-DeliverIN (OZ Biosciences, cat. #P110250) according to manufacturer’s protocol. Per well of a 96-well plate 0.25 µl of Pro-DeliverIN was mixed with 0.75 µg Nb and incubated for 15 min at RT. Volume of 20 µl Opti-MEM (Thermo Fisher Scientific, cat. #31985062) was added to the mixture and immediately transferred to the cell culture medium in the well. After 2 h, medium was replaced with imaging medium DMEMF-2 (Evrogen, cat. #MC102) supplemented with 10% FCS, 2 mM l-glutamine and cells were imaged.

Live-cell staining and imaging. HeLa Kyoto transiently expressing bc2-GFP-GPL, HeLa-Bc2-GFP-ABC, or A549-Bc2-ABC cells were plated at ~5000 cells per well of a 96-well plate (Greiner Bio One, cat. #655900) and cultured at standard conditions. Next day, time-lapse imaging was performed in a humidified chamber (37 °C, 5% CO₂) of a MetaXpress Micro XL system (Molecular Devices) at x40 magnification. For live-cell staining of bc2-GFP-GPL, culture medium was replaced without washing by live-cell visualization medium DMEMF-2 (Evrogen, cat. #MC102) supplemented with 10% FCS, 2 mM l-glutamine and 1 µg/ml bc2-NA647. Time-lapse imaging with 4–5 min intervals was started immediately upon medium replacement. For live-cell staining of HeLa-Bc2-ABC and A549-Bc2-ABC upon protein transduction of nanobodies, cells were washed once with and placed in DMEMF-2 medium 2 h after addition of transduction mix (see "protein transduction" section above) and imaged in hourly intervals.

Quantification of staining intensities. HeLa Kyoto cells were plated at ~5000 cells per well of a 96-well plate (Greiner Bio One, cat. #655900) and transfected with expression plasmid for mCherry-VIM-BC2T. Next day, cells were fixed and stained with the same concentration (1 µg/ml) of monovalent or bivalent BC2-Nbs conjugated to AF647 either by NHS conjugation or via sarsite. Thus, the intensity of the fluorescence staining was calculated by the ratio of the staining intensity in mCherry-BC2T expressing cells and in non-transfected cells (background). Staining intensities were determined using a
custom-written cell identification algorithm (MetaXpress, Custom module editor). In brief, transfected cells were identified based on cell size parameters and a threshold setting for mCherry fluorescence intensity above local background. Background fluorescence was defined as the average fluorescence of the remaining image area precluding mCherry-VIMaC2 expressing cells. For statistical significance the average fluorescence intensity of a large number of transfected cells was determined (BC2- NbAF647 (Intens.) \( n = 115 \); bivBC2-NbAF647 (Intens.) \( n = 134 \); BC2-NbAF647 (Area) \( n = 130 \); bivBC2-NbAF647 (Area) \( n = 193 \)).

**Immunofluorescence staining for dSTORM imaging** To achieve the higher labeling density required for dSTORM imaging the staining protocol was slightly modified. Cells were prepared the same way as described up to the storage step in PBS. After storage, cells were blocked with 10% (w/v) BSA (Carl Roth, cat. #8076) in PBS for 30 min, then additionally with Image-iT FX signal enhancer (ThermoFisher Scientific, cat. #A-31571). For staining with nanobodies, cells were incubated at 4 °C for 4 h with the primary antibody (V9, mouse monoclonal, Sigma-Aldrich, cat. #A32331) followed by two washes with PBS, and then stained at 4 °C for 24 h with the secondary antibody (donkey-anti-mouse AF647, ThermoFisher Scientific, cat. #A-13067). For staining with antibodies, cells were incubated at 4 °C for 4 h. Unbound Nbs were removed by two washes with PBS-T (0.1% w/v Tween-20 (Sigma-Aldrich, cat. #P7949)) and samples were post-fixed with 4% PFA (Sigma-Aldrich, cat. #P8877) and 0.25% (w/v) glutaraldehyde (Sigma-Aldrich, cat. #G5882) in PBS for 5 min to make the binding permanent. Finally, cells were washed twice with PBS to remove fixation solution and stored in PBS with 0.1% (w/v) sodium azide (Carl Roth, cat. #4221) until imaging.

dSTORM imaging and post-processing. A 1:5000 dilution of fluorescent beads (FluoSpheres 715/755, ThermoFisher Scientific, cat. #F8799) was sonicated to break up clumps of beads. Volume of ~5 µl of the beads were added to the sample and allowed to settle and adhere for 15 min, to serve as fiducial markers for drift correction. Images were recorded on a customized Nikon Ti-Eclipse inverted microscope, equipped with a CFI Apochromat TIRF x100 objective with a numerical aperture of 1.49 (Nikon) and an iXON ULTRA 888 EMCCD camera (Andor). AF647 was imaged in 100 mM MEA (Sigma-Aldrich, cat. #M56000-25G) with a glycolic acid (Sigma-Aldrich, cat. #G2113, C100) oxygen scavenging system\(^3\). The sample was illuminated with an OBIS LS 637 nm laser (Coherent) which was filtered through a ZET 640/10 bandpass, modulated by an Acousto-Optic Tunable Filter (GeoCh & Housso, TF25-250-6-3G18) and focused by a ZET405/488/561/640 m dichroic mirror (Chroma) onto the back focal plane of the objective resulting in a final intensity of 2–4 kW cm\(^{-2}\) in the sample. The readout was collected by blocking the laser light by the bandpass ZET405/488/561/640 and passing through a 689/29 nm single-band bandpass filter (All filters AFH Analyten- sentech AG). For each dSTORM image reconstruction, 10,000–20,000 imaging frames with an exposure time of 70 ms were recorded at a pixel size of 129 nm. The camera, microscope and AOTF were controlled by pManager software on a PC workstation. Single-molecule localizations were extracted from the movies with the open-source software Rapidstorm 3.2.\(^3\) Drift correction was performed by custom written Python 2.7 algorithms that extract and correct for fluorescent bead tracks. NeNa as described in ref. \(^6\) was done with the open-source software Luma\(^7\) on a section of the image that contained no fiducial markers. Localizations appearing within the radius of the NeNa value on several frames were grouped into one localization using the Kalman tracking filter in Rapidstorm 3.2. Final images were reconstructed at a pixel size of 10 nm. For visualization, a Gaussian blur filter was applied in the ImageJ software using NeNa as the sigma value. dSTORM imaging in yeast cells was performed in PEL buffer containing 10 mM MEA (Sigma-Aldrich, M65000) and 1 mM methyl viologen dichloride hydrate (MV) (Sigma-Aldrich, cat. #856177).

**PALM imaging and post-processing.** For imaging of ParnCherry-vimentin, the sample was illuminated with an OBIS LS 561 laser (Coherent), filtered through a ZET 561/10 clean-up filter (AHF Analytensentech, Germany) at an intensity of 800 W cm\(^{-2}\) and an OBIS LS 405 laser (Coherent) at intensities of 350 W cm\(^{-2}\) in the center and 200 W cm\(^{-2}\) at the edge. The readout was collected through a 610/75 bandpass filter (AHF Analytensentech, Germany). Twenty thousand imaging frames were recorded at an exposure time of 70 ms. The number of ParnCherry molecules activated each frame was kept at a steady rate by increasing 405 laser intensity until all of the ParnCherry was readout. All other microscope parameters and image post-processing remained the same as for dSTORM imaging.

**Super-resolution analysis of endogenous β-catenin staining.** To evaluate the effect of endogenous β-catenin staining on dSTORM imaging of low-abundance non-structural proteins, non-transfected HeLa and BC2-LC3B expressing HeLa cells were fixed and stained with bivBC2-NbAF647 or GFP-NbAF647 for 48 h, imaged and post-processed as described in the “Immunofluorescence staining for dSTORM imaging” and “dSTORM imaging and post-processing” sections. Localization counts for three cells per condition were obtained with the RapidSTORM software\(^9\) and cell areas were measured in Fiji.\(^7\) Localizations per µm\(^2\) were counted and the means of each condition plotted as bar charts with S.D. as the error.

**Imaging of BC2-Ypet-Fna and SNAP-Fna lysates.** A 488 nm Sapphire laser (Coherent Inc., Santa Clara, California USA) was used to excite the Ypet and the readout was collected through a 525/50 single bandpass filter (AHF Analyten- sentech for the occurrence of each labeling spot (fig. 24, 22, 23... AF647 per oligomer). Data sets assuming full labeling were simulated by calculating the linear combination of calibration distributions following the binomial mixture of visual oligomeric states due to the degree of labeling by recursively convolving the single-fluorophore log-normal distribution (μ = 5.68, σ = 0.4) using a Matlab (MathWorks, Natick, MA-US)-based tool, as published and described in ref. \(^28\). To evaluate the completeness of labeling with the bivBC2-NbAF647 nanobody and the SNAP-tag, we then compared the medians of the simulated fully labeled scenario data sets with our measured distributions.

**Image analysis of dSTORM images.** A custom ImageJ script was used to measure the widths and coverages of vimentin, actin, and tubulin fibers on reconstructed non-blurred images. The segmented line tool in ImageJ was used to manually draw lines along vimentin filaments and line thickness was adjusted to fit the filament. Each image was divided into 10 × 10 µm sections and 15 random filaments were measured in each section. Line selections were straightened using the straighten tool and an intensity profile was plotted for each filament. To determine filament width, the intensity profile was fitted with a Gaussian curve and the resulting sigma value was multiplied with 2.35 to obtain the full width at half maximum (FWHM). To determine lengthwise coverage the image was converted into a binary image and the fraction of covered area of the middle 3 pixels was calculated. To reduce measurement error, line selections were wobbled by 0.5 pixels in four directions and the result was averaged. To estimate image resolution, custom written Python 2.7 (Python Software Foundation) and Fiji\(^7\) algorithms using code from the Lama software\(^56\) and GDSC SMLM ImageJ plugin (http://
NeNA localization precision and Fourier Image Resolution (FIRE) were calculated for all analyzed regions. To calculate FIRE localization files were split into two by alternating frames (“random split” option unchecked). The threshold was set to Fixed 1/7 and the Fourier image scale was set to a constant 16 for all analyzed regions. Other parameters were left as default (auto image scale = 2048, sampling factor = 1). The results were plotted using the software OriginPro (Origin Lab Corp.).

LC3B clustering analysis. Imaging and post-processing of B2C-LC3B cells was done as described in the “dSTORM imaging and post-processing” section. The obtained localization files were loaded into the Matlab-based software PALMsiever. Density-based clustering analysis was performed with the density-based spatial clustering of applications with noise (DBSCAN) algorithm (DBSCAN parameters: eps = 50 nm; MinPts = 40). Three cells were analyzed for the untreated conditions and four cells for the rapamycin-treated conditions. To compare the degree of clustering, the ratio of non-clustered to clustered localizations was calculated for each cell. Points assigned as core and border points by DBSCAN were considered as clustered, while noise points were considered as non-clustered. Cluster size was calculated with a custom-written Python 2.7 script, measuring the average distance of cluster points from the cluster center of mass. Degree of clustering results were plotted as stacked bar charts, using S.D. as the error and the populations.

Data availability. The data sets that support the findings of this study are available from the corresponding authors upon reasonable request.

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**Author contributions**
U.E. and U.R. conceived the study. D.V., B.T., J.M., P.D.K., M.B., C.S., I.V., B.P.-L., U.E., and U.R. performed all experiments. U.E. and U.R. wrote the manuscript with the input from all authors.

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