Supplementary Information

Supplementary Discussion

It is generally known that wild-type avidin is capable of binding to four biotin molecules with high affinity (dissociation constant is $10^{-15}$ M).\textsuperscript{1} Although scAVD has a similar biotin binding capacity and dissociation rate constant as wild-type avidin,\textsuperscript{2} in our \textit{in vitro} study, probes with less than four fluorophores were also observed. This phenomenon also existed in the streptavidin sample that was a positive control with four functional high-affinity biotin-binding sites (dissociation constant is $10^{-14}$ M). One possible reason of this phenomenon is fluorophore photobleaching during focus adjustment. Another is the dilution of probe concentrations during the purification process. Although the protein concentration in the reaction mixture is $\sim 10^{-5}$ M, which is much higher than the dissociation constant, the protein concentration might be highly diluted during the purification process. It is possible that dissociation of biotin occurs during purification and probes with less than four fluorophores are therefore generated.

The cellular permeability of fluorescent organic dyes is a critical factor for single-molecule observation in our system. TAMRA is a rhodamine-based dye with superior cellular permeability, while Cy5 is recognized as impermeable to plasma membrane due to the hydrophilic sulfonate groups.\textsuperscript{3} After azide functionalization, TAMRA still exhibited good cellular permeability (Fig. S5).\textsuperscript{4} Cy5 also showed plasma membrane permeability to certain extent after azide modification (Fig. S3). TAMRA probes exhibited an even distribution on the organelles where they were assembled (Fig. 5a), whereas Cy5 probes distributed as separate dots (Fig. 3a). This is probably because Cy5-azide is poorer in cellular permeability than TAMRA-azide, leading to a lower population of Cy5-azide dyes inside a cell and thereby allowing
observation of discrete Cy5 dots. To expand our strategy using a variety of fluorescent dyes and further achieve the observation of single probes, the cellular permeability should be taken into account.

Concern on the cellular permeability of dyes prompt us to use the three-step approach rather than a simpler two-step approach that biotin-conjugated dyes directly react with scAVD to form nanoprobe. Biotin is a hydrophilic molecule. Conjugation of biotin to fluorescent dye may lead to poorer permeability to cells. Comparatively, azide moiety is small and hydrophobic. We expected that conjugation with azide may not compromise the cellular permeability of the fluorescent dye. However, we do not exclude the possibility that there are biotin conjugates with high cellular permeability, such as the DBCO-biotin that was used in this study. DBCO moiety is substantially hydrophobic and is comparatively smaller in size than fluorescent dye, which may contribute to the cellular permeability of DBCO-biotin.

The diameters of both mCherry (~ 27kD) and scAVD (~ 63 kD) are less than 3 nm. The sizes of single organic molecule of DBCO-biotin (~ 750 D) and Cy5-azide (~ 830 D) are less than 1 nm. Therefore, we consider that the diameter of a single Cy5 dot (mCherry-scAVD-(biotin-DBCO-azide-Cy5)) is less than 10 nm. The small size of nanoprobe is essential for revealing the true locations of biomolecules or tracking the accurate remodeling trajectories of biological structures without affecting their functions.
Supplementary Methods

Materials

Calcein AM, pHrodo dextran green, CellMask green plasma membrane stain and MitoTracker green were purchased from Invitrogen.

Cell viability assay

Cells were incubated with 1 µM Calcein AM together with DBCO-biotin or Cy5-azide or TAMRA-azide at the concentration of 0.1 µM, 1 µM, 10 µM or 100 µM, for 1 hour at room temperature. Following three times wash with PBS, cells were observed under epifluorescence microscope. Cells with Calcein fluorescence were considered as alive cells. Viability = number of alive cells/number of total cells × 100.

Confocal microscopy

For staining plasma membrane, mCherry-scAVD overexpressing cells were incubated with CellMask at room temperature for 10 min with 1:1000 dilution after incubation with Cy5-azide. For staining endosomes, mCherry-scAVD overexpressing cells were incubated with 40 µg/ml pHrodo Green Dextran together with Cy5-azide for 1 hour at either 37 °C or room temperature (22 °C). Microscopic images of CellMask and pHrodo Green Dextran were obtained with the FITC filter set (488-nm laser) on Olympus confocal microscope (FluoView FV1000).

Epifluorescence

For observing mitochondrial localization of TAMRA-azide, cells were incubated with 100 nM MitoTracker green for 15 min at room temperature after incubation with TAMRA-azide.
Calcein staining images were acquired with an UPlanSAPO 10× NA0.40 objective lens, a BP470-495 excitation filter, a DM505 dichroic mirror and a BP510-550 emission filter (all from Olympus).

**In vitro spectroscopy**

Fluorescence spectra of TAMRA-azide were measured using a F-2700 fluorescence spectrophotometer (Hitachi). Excitation wavelength was 540 nm. The slit width was 2.5 nm. The dye concentration is 1 µM in PBS (pH 7.4). The cuvette containing dye solution was heated in an oven to 60 °C and then put inside the spectrophotometer. The fluorescence spectra were recorded at every 2 °C from 40 °C to 32 °C when the temperature gradually dropped down. The temperature of solution in cuvette was measured by a digital thermometer (RX-450K, AS ONE, Japan) with an accuracy of ± 0.1 °C.

**Determination of the temperature sensitivity of TAMRA-azide in fixed HeLa cells**

HeLa cells were treated with 100% ethanol at -20 °C for 10 min after incubation with TAMRA-azide. By changing the temperature in the buffer with a stage top incubator (the actual temperature was monitored by a digital thermometer), fluorescence intensity of TAMRA-azide at different temperatures in a whole cell was measured.
**Supplementary Figures**

Fig. S1 Photobleachings of dots with less than four fluorophores generated *in vitro* in scAVD sample. (a-c) Time courses of fluorescence intensity changes of dots with three (a), two (b) or one (c) fluorophore(s) during photobleaching. Orange arrows indicate the photobleaching steps.
Position displacements of dots were also shown (time points that cannot be tracked by software were omitted). Dashed lines separate different photobleaching stages. Values on top of graphs indicate the localization precision at each stage.
Fig. S2 Evaluation of the toxicity of DBCO-biotin, Cy5-azide and TAMRA-azide in HeLa cells.

The viability of cells was evaluated by Calcein AM staining. Data are mean ± SD (n = 3).
**Fig. S3** Cy5-azide can pass through plasma membrane by diffusion. (a) Confocal fluorescence images of mCherry-scAVD overexpressing cells stained with CellMask green. (b) Confocal fluorescence images showing the colocalization of Cy5-azide and pHrodo Green Dextran. Scale bars, 10 µm.
Fig. S4 (a and b) Histograms showing the distribution of the instant velocities of static dots (n = 30) and moving dots (n = 49). A dot is considered as a static dot if all instant velocities within 88 s is below 30 nm/s; otherwise, it’s considered as a moving dot.
**Fig. S5** TAMRA-azide can efficiently pass through plasma membrane. Epifluorescence images of HeLa cells stained with both MitoTracker green and TAMRA-azide. TAMRA-azide naturally localized to mitochondria in HeLa cells at an extremely low level. Scale bar, 10 µm.
Fig. S6 Evaluation of TAMRA-azide temperature sensitivity in cuvette. (a) Emission spectra of TAMRA-azide at different temperatures. (b) Fluorescence intensity at 580 nm at different temperatures was normalized to that at 36 °C and plotted versus temperature. Data are mean values ± SD (n = 3 independent measurements).
Fig. S7 Evaluation of the temperature sensitivity of TAMRA-azide in fixed HeLa cells. (a)

Epifluorescence images of fixed HeLa cells overexpressing either EGFP or EGFP-scAVD. Scale
bar, 20 μm. (b) Fluorescence intensities of TAMRA-azide at different temperatures (300 ms exposure time) in cytosol in fixed scAVD-overexpressing cells was normalized to 37 °C. The temperature sensitivity of TAMRA-azide, −2.1 %/°C, was determined by plotting the relative fluorescence intensity versus temperature. Data are mean ± SD (n = 11 cells). (c) Cells were imaged over 100 s using an epifluorescence microscope with the same conditions as used for b, and the fluorescence intensity at each time point was normalized to the first time point.
Fig. S8 Evaluation of ER tracker temperature-sensitivity on ER in living Hela cells. (a) The same HeLa cell shown in Fig. 5b was stained with ER tracker. Regions (colored circles) with different distances from the heating spot (white circle) were chosen and analyzed in b. Scale bar, 10 µm.
(b) ‘Square wave’ was not generated in response to laser heating and re-cooling. (c) Under the same laser power in the same cell as in Fig. 5b, ER tracker green cannot illustrate the temperature map because of its low thermosensitivity compared with TAMRA-azide. Scale bar, 10 µm. (d) With the same calculation method as Fig. 5e, temperature sensitivity of ER tracker green at $-0.46 \%/\degree \text{C}$ was determined (24 cells were analyzed).
Supplementary Movie Legends

**Movie S1.** Photobleaching movie of a dot with four Cy5-azide dyes in scAVD sample *in vitro*, corresponding to the time course curve in Fig. 2c. The total duration is 300 s. 2.65 µm × 2.65 µm (W × H), created using the Walking Average plug-in of ImageJ with 4 frames averaged.

**Movie S2.** Photobleaching movie of a dot showing four steps of fluorescence intensity decrease in cells with scAVD overexpression, corresponding to the time course curve in Fig. 3b. The total duration is 90 s. 2.65 µm × 2.65 µm (W × H), created using the Walking Average plug-in of ImageJ with 4 frames averaged.

**Movie S3.** Photobleaching movie of a dot showing gradual fluorescence intensity decrease without clear steps in cells with scAVD overexpression, corresponding to the time course curve in Fig. 3c. The total duration is 90 s. 2.65 µm × 2.65 µm (W × H), created using the Walking Average plug-in of ImageJ with 4 frames averaged.

**Movie S4.** Photobleaching movie of a single Cy5-azide fluorophore located on a glass bottom dish outside cells, corresponding to time course curve in Fig. 3d. The total duration is 90 s. 2.65 µm × 2.65 µm (W × H), created using the Walking Average plug-in of ImageJ with 4 frames averaged.
Supplementary Notes and references