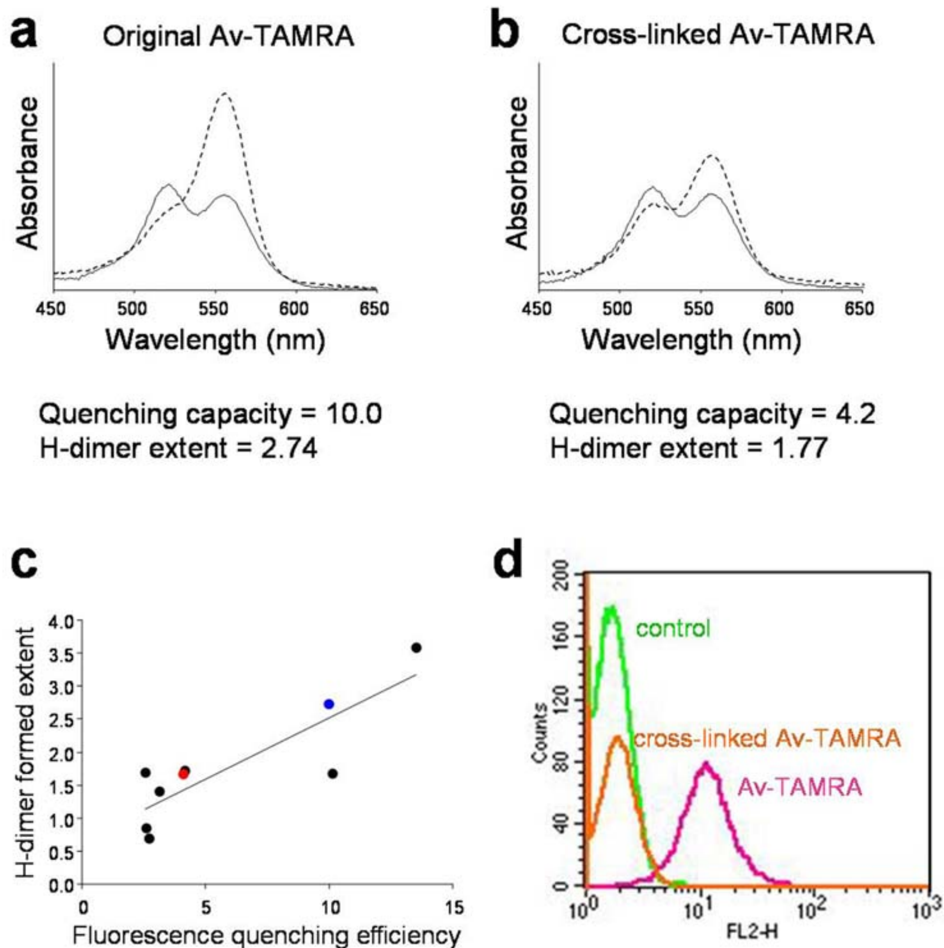

Supplemental Figure 1

To confirm that limiting conformational changes of the carrier protein supresses H-dimer dissociation and fluorescent activation, avidin tetramer was cross-linked by disuccinimidyl suberate (DSS; Pierce, Rockford, IL) to minimize the dissociation and the conformational

changes of avidin in the lysosome. Absorbance spectrums of Av-TAMRA (a) and cross-linked Av-TAMRA (b) without SDS (solid line) and with SDS (dashed line) conditions indicate that H-dimer dissociation was inhibited by cross-linking of the avidin tetramer. Both quenching capacity and dissociable H-dimer formation were lower in the cross-linked probe. The results of quenching efficiency and H-dimer formation for cross-linked Av-TAMRA were plotted as a red dot on the correlation graph (c). The blue dot shows the results for Av-TAMRA. The slope of correlation line without the red dot is 0.186. This is similar to the slope of the line connecting the red and blue dots, 0.167. These results show that the inhibition of H-dimer dissociation by cross-linking of avidin significantly contributes to the decrease in fluorescence activation, in other words, the fluorescence activation is attributed to the conformational change of the carrier protein. FACS flow cytometry results are shown in (d). SHIN3 cells were incubated with Av-TAMRA (10 μg/mL) or cross-linked to Av-TAMRA (10 μg/mL) for 10hrs at 37°C. The mean values were 1.6, 10.9 and 1.8 for control, Av-TAMRA and cross-linked Av-TAMRA, respectively. The cross-linked Av-TAMRA showed minimum fluorescence, due to a failure of fluorescence activation, which is concordant with the results above.

Supplemental Figure 2

a

14540.88 ← Avidin monomer

+ 1 fluorophore → 14952.50

15764.00 ← Avidin monomer (K⁺ adduct)

+ 2 fluorophores → 15558.18

16173.78 ← + 1 fluorophore (K⁺ adduct)

16588.42 ← + 2 fluorophore (K⁺ adduct)

b

Supplemental Figure 2

MALDI Mass spectra of Av-TAMRA were performed using a MALDI-TOF-TOF system (Axima Performance, Shimadzu, Columbia, MD) using linear positive ionization mode equipped with a 337 nm nitrogen laser operating at 50 Hz. All samples were spotted using the dried droplet method using 1 μl sample and 1 μl matrix. In tact protein samples were prepared using purified Sinapinic Acid (20 mg/ml in 50% Acetonitrile, 50% H2O, 0.1%

TFA) (Laser BioLabs, Sophia-Antipolis, France). In source decay analysis was performed using 1,5-diaminonapthalene (10 mg/ml in 50% Acetonitrile, 50% H2O, 0.1% TFA) (Protea Biosciences, Morgantown, WV). Similar heights of peaks were obtained with TAMRA conjugated avidin monomer in differing ratios (1:1 and 2:1) peaks (a). In addition, TAMRA conjugated lysine residue (90) was detected by further analysis (b).

Supplemental Videos

***In vivo* real-time endoscopic videos of the SHIN3 tumor visualized with either Av-Alexa488 (A; "always-on", with minimal H-dimer formation) or Av-TAMRA (B; "quenched/activatable", with H-dimer formation). Fluorescence-guided endoscopic tissue biopsy was performed with injection of Av-TAMRA (C).**

These endoscopic videos show that peritoneal tumor implants, which were originated form the same ovarian cancer SHIN3 cell lines, emit different fluorescence signals from pre-injected Av-Alexa488 (**A**; "always-on", with minimal H-dimer formation) and Av-TAMRA (**B**; "quenched/activatable", with H-dimer formation) reagents 2 hrs before imaging. The tiny tumors were clearly visualized with the activatable probe, Av-TAMRA with H-dimer formation (see video **B**). In contrast, Av-Alexa488, an always-on probe with minimal H-dimer formation, showed high background signal and high fluorescence from unabsorbed injected fluid in the peritoneal cavity (see video **A**).

Left: image without emission filter, right: fluorescence image through an appropriate emission filter. First half: excitation light image specific for Alexa488 (**A**) and TAMRA (**B**), Second half: white light image.

In addition, we performed tissue biopsy from 50 places with or without fluorescence signal (25 in each) under fluorescence-guided endoscope (see video **C**).