Published in final edited form as:

Anal Chem. 2009 February 1; 81(3): 883–889. doi:10.1021/ac801932m.

Fluorescent avidin-bound silver particle:

a novel strategy for single target molecule detection on cell membrane

Jian Zhang¹, Yi Fu¹, Dong Liang², Richard Y. Zhao^{2,3,4}, and Joseph R. Lakowicz¹

¹Center for Fluorescence Spectroscopy, University of Maryland School of Medicine, Department of Biochemistry and Molecular Biology, 725 West Lombard Street, Baltimore, MD 21201

²Division of Molecular Pathology, Department of Pathology, University of Maryland School of Medicine, 10 South Pine Street, Baltimore, MD 21201

³Division of Molecular Pathology, Department of Microbiology-Immunology, University of Maryland School of Medicine, 10 South Pine Street, Baltimore, MD 21201

⁴Institute of Human Virology, University of Maryland School of Medicine, 10 South Pine Street, Baltimore, MD 21201

Abstract

Cy5-avidin conjugate-bound silver nanoparticles were prepared as fluorescence molecular reagent for the cell imaging. Compared with the metal-free avidin conjugate, the avidin-metal complex was observed to display a stronger emission intensity, shorter lifetime, and better photostability. The avidin-metal complexes were conjugated with the biotin-sites on the surfaces of PM1 cell lines and the cell images were recorded using scanning confocal microscopy. It was noticed that the avidin-metal complexes bound on the cell surfaces could be identified as the isolated emission spots distinct from the cellular autofluorescence. The emission intensity over the cell image was increased with an increase of the number of avidin-metal complex on the cell surface but the lifetime was decreased. A quantitative regression curve was achieved between the amount of avidin-metal complex on the cell surface and the emission intensity or lifetime over the entire cell image. Based on this curve, we expect to develop an approach that can be used to quantify the amount of target molecules on the cell surfaces using the cell intensity and lifetime images at the single cell level.

Keywords

silver nanoparticle; Cy5; avidin; T-lymphocytic (PM1) cells; avidin-metal complex; scanning confocal microscopy; fluorescence intensity; lifetime image

Fluorescence technology is being used widely in biological detection and clinical diagnosis [1,2]. In the past decade, fluorescence single molecule detection (SMD) has been developed to investigate fluorophores with the heterogeneous spectral properties at the single molecule level [3,4]. The SMD technique can also be used to detect the single target molecules from the fluorescent cell images that elucidate signaling pathways and perform disease diagnosis [5, 6]. In the imaging measurements, the cell lines are fluorescently labeled by molecular imaging reagents that consist of fluorophore moieties, e.g. organic fluorophores in most cases, and targeting functionalities, e.g. antibodies, peptides, DNAs, or special ligands [7-10]. However, the convenient organic fluorophores usually have their disadvantages, e.g. low emission intensity to background of cellular autofluorescence, poor photostability, and strong

photoblinking, etc [11,12]. In addition, the lifetimes of most organic fluorophores are in the range from 2 to 5 ns, at which the cell lines display strong cellular autofluorescence [13]. As the result, the identification to the emission signals from the bound probes becomes difficult. Therefore, it is highly interesting to develop the novel molecular fluorophores with strong emission signals and different lifetimes for use in cell image measurements.

It is known that when localizing a fluorophore near a metal particle with a sub-wavelength size, the emission properties can be altered dramatically [14-17]. This change occurs via a near-field coupling interaction of the fluorophore with the metal particle [18,19]. It appears that the fluorophore acts as an oscillating dipole and couple with the plasmon resonance in metal particle. This system radiates to the far field. In the coupling interaction, the radiative rate of fluorophore is increased by the plasmon resonance from the metal particle, leading to an increase of apparent intrinsic decay rate [20]. This phenomenon is defined as metal-enhanced fluorescence (MEF). MEF can cause a significant enhancement of emission intensity that is accompanied with a shortening of lifetime, an increase of photostability, and a reduction of photoblinking [21-24]. Based on these features, we are developing a new class of probes consisting of fluorophores bound on the metal nanoparticles, which can satisfy the requirements to the novel molecule reagents in the cell imaging. Recently, these metal probes were used to bind on the cell surfaces to monitor the cell images in either the emission intensity and lifetime [25,26]. It was revealed that the emission signals from the metal probes could be isolated and counted on the cell images especially on the lifetime images. In this paper, we plan develop an approach to using these metal probes that can be used to quantify the number of target molecules on the cell surfaces from the single cell images. The PM1 cell lines were biotinylated by EZ-link sulfo-NHS-biotin reagent and the number of biotin site on the cell surface was controlled by the concentration of biotin reagent in the reaction.

Fluorophore-avidin conjugates are widely used as secondary antibody reagents to bind with the target molecules on the cell surfaces for the cell imaging [27-30]. In this study, we bound the Cy5-avidin conjugates on the silver particles to prepare the metal fluorescence probes. Although the larger silver particles can bring up a more efficient MEF to a nearby fluorophore [22], we use the silver particles with an average diameter of 20 nm in this case in order to reduce the possible steric hindrance from them when being proximate to and interacting with the biotin targets on the cell surfaces [31]. These silver particles were protected with N-(2-mercaptopropionyl)glycine (abbreviated as tiopronin) for their good water solubility and chemical stability [32,33]. Cy5, a typical organic fluorophore for SMD measurement, was used as fluorophore to conjugate with the avidin molecule [34,35]. Besides the efficient MEF [22], Cy5 can typically emit the signal at a wavelength close to the optical window for the biological detection [36]. In this stud, we bound Cy5-avidin metal complexes on the surfaces of biotinylated PM1 cells via biotin-avidin interactions [37-39]. The fluorescence cell images were recorded in the emission intensity and lifetime using scanning confocal microscopy. A quantitative regression curve was created to analyze the relation between the number of bound fluorescence probe and the emission intensity or lifetime over the entire cell images.

Experimental section

All reagents and spectroscopic grade solvents were used as received from Sigma-Aldrich. EZ-link sulfo-NHS-biotin was commercially available from Pierce. Cy5-avidin conjugates were from Invitrogen. PM1 cell line was obtained through the AIDS Research and Reference Reagent Program, the National Institute of Health (NIH). RC dialysis membrane (MWCO 50,000) was purchased from Spectrum Laboratories, Inc. Nanopure water ($>18.0 \text{ M}\Omega\text{cm}^{-1}$) purified using Millipore Milli-Q gradient system, was used in all experiments. (2-mercaptopropionylamino) acetic acid 2,5-dioxo-pyrrolidin-1-ylester was synthesized as we reported previously [40].

Preparation of avidin-metal complex

Tiopronin-coated silver particles were prepared by a modified Brust method in a molar ratio of tiopronin/silver nitrate = 1/6 in methanol with an excess amount of sodium borohydride as reducing reagent [20]. The tiopronin-coated silver particles were succinimidylated *via* ligand exchange reaction [33], in which (2-mercapto-propionylamino) acetic acid 2,5-dioxo-pyrrolidin-1-ylester (1×10^{-6} M) and silver particle (5×10^{-8} M) were co-dissolved in a mixing solvent of water / ethanol (v/v = 1/1) and stirred for 72 h at room temperature. Unbound compounds were removed by centrifugation. The residual solid was washed with ethanol and water, respectively, and then redispersed in 10 mM PBS buffer solution at pH 7.2.

Cy5-avidin conjugates were covalently bound on the metal particles [37]. In the experiment, the metal particle (2×10^{-8} M) and Cy5-avidin conjugate (2×10^{-5} M) were co-dissolved in 10 mM PBS buffer solution and incubated for 2 h. The solution was centrifuged at 2,000 rpm for 5 min to remove the possible metal particle aggregates, and then further centrifuged at 6,000 rpm for 30 min to precipitate the avidin-metal complexes. After removing the suspension, the solid residue was redispersed in 10 mM PBS solution at pH 7.2. A drop of ammonium was added in solution to consume the residual succinimidyl ester moieties on the metal particles, and then the avidin-metal complexes were precipitated again by centrifuging at 6,000 rpm, washed with buffer solution, and dispersed in 10 mM PBS solution.

Cell culture

T-lymphocytic *PM1* cell line, which is a colonial derivative of HUT 78, was separated by ficoll-hypaque density gradient centrifugation [41]. They were grown in RPMI-1640 culture medium (Sigma) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Atlanta Biologicals Inc. GA) and contained 200 units/ml penicillin, 200 units/ml streptomycin (Invitrogen) and recombinant human interleukin (100U/ml) (Roche, Indianapolis, Indiana, USA) for 6 days prior to fluorescent labeling. The number of cells was counted to be *ca.* 5×10^6 cells / ml.

Biotinylation and labeling of cell lines

PM1 cells were treated in culture solution with 10^{-2} , 3×10^{-2} , 10^{-1} , 3×10^{-1} , 1, 3, 10 nM EZ-link sulfo-NHS-biotin, respectively, for 30 min [38,39]. Washing with 10 mM PBS buffer solution (pH = 7.2), the surface-biotinylated cell lines were suspended in 500 μ L 10 mM PBS buffer solution and labeled with 1 μ M metal free Cy5-avidin conjugate or 0.1 μ M avidin-metal complex for 1 h [42]. All cell samples were washed at least three times with 10 mM PBS buffer solution before casting them on the glass coverslips for the fluorescence image measurements.

Spectra and image measurements

Absorption spectra were monitored with a Hewlett Packard 8453 spectrophotometer. Ensemble fluorescence spectra were recorded with a Cary Eclipse Fluorescence Spectrophotometer. Transmission electron micrographs (TEM) were taken with a side-entry Philips electron microscopy at 120 keV. Samples were cast from water solutions onto standard carbon-coated (200-300 Å) Formvar films on copper grids (200 meshes) by placing a droplet of a 10 times diluted aqueous sample solution on grids. The size distribution of metal particles was analyzed with Scion Image Beta Release 2 counting at least 200 metal particles.

All cell image measurements were performed using a time-resolved confocal microscope (MicroTime 200, PicoQuant) [43]. The samples were immobilized on glass coverslips by adding 20 μ L suspension onto an amino-silanized coverslip following by drying at room temperature. A single mode pulsed laser diode (635 nm, 100ps, 40 MHz) (PDL800, PicoQuant) was used as the excitation light. The collimated laser beam was spectrally filtered by an excitation filter (D637/10, Chroma) before directing into an inverted microscope (Olympus,

IX 71). An oil immersion objective (Olympus, 100 $\times$, 1.3NA) was used to focus laser light and collect fluorescence signal. The fluorescence that passed a dichroic mirror (Q655LP, Chroma) was focused onto a 75 μ m pinhole for spatial filtering to reject out-of-focus signals and then reached the single photon avalanche diode (SPAD) (SPCM-AQR-14, Perkin Elmer Inc). Images were recorded by raster scanning (in a bidirectional fashion) the sample of the incident laser with a pixel integration of 0.6 ms. The excitation power into the microscope was maintained at less than 1 μ W. Time-dependent fluorescence data were collected with a dwell time of 50 ms. The data was stored in a time-tagged-time-resolved (TTTR) mode, which allows recording every detected photon with its individual timing information. Instrument Response Function (IRF) widths of about 300 ps FWHM were obtained in combination with a pulsed diode laser, which permits the recording of sub-nanosecond fluorescence lifetimes extendable to less than 100 ps with reconvolution. Lifetimes were estimated by fitting to a χ^2 value of less than 1.2 and with a residuals trace that was fully symmetrical about the zero axis. The image measurements were performed in a dark compartment at room temperature.

Results and Discussion

The tiopronin-coated silver particles were synthesized with an average diameter of 20 nm and the size distribution appeared in a range 20 ± 8 nm [22]. The absorbance spectrum of silver particles displayed a typical metal plasmon resonance at 406 nm (absorbance coefficient = 1.5×10^7 M⁻¹cm⁻¹) [31]. In order to bind the Cy5-avidin conjugates, the metal particles were succinimidylated *via* ligand exchange, in which the tiopronin ligands coated on the metal core was displaced with (2-mercapto-propionylamino) acetic acid 2,5-dioxo-pyrrolidin-1-ylester in a molar ratio of 1:1 [40]. Because the metal particle and succinimidyl ester compound were co-dissolved in the reaction solution at a molar ratio of 1:20, the loading number of succinimidyl ester ligand was estimated to be less than 20 per metal particle.

Cy5-avidin conjugates were bound on the silver particles. In order to avoid the aggregation of metal particle, the avidin conjugate was in 100-fold excess amount in the reaction solution. The avidin metal complexes displayed a typical metal plasmon resonance at 410 nm on the absorbance spectrum slight shifting from 406 nm of the tiopronin-coated silver particles, indicating that the metal particles were not influenced significantly by the two-step surface reactions on the metal particles. Simultaneously, a small rise near 640 nm was observed, which was from the bound Cy5-avidin conjugates, indicating that the Cy5-avidin conjugates were bound on the metal particles. On the TEM images of avidin-metal complexes, most metal particles were observed to exist as individual spots confirming that the metal particles were not aggregated by the avidin molecules. Upon the excitation at 630 nm, the avidin-metal complexes displayed the emission band at 668 nm close to the metal-free avidin conjugates. But the emission band of avidin-metal complexes was noticed to be broadened significantly, which was probably due to the movement restriction to the bound fluorophores on the metal particles [44].

The number of bound avidin molecule per metal particle was estimated in the NaCN treatment as described previously [11]. Typically, a drop of 0.1 N NaCN solution was added into the metal particle solution. The metal cores of particles were dissolved, and simultaneously, the bound avidin conjugates were released as free. In this case, the concentration of released avidin conjugate could be estimated accurately by the emission intensity of emission spectrum and the concentration of metal particle could be estimated by the absorbance. We estimated the ratio of avidin conjugate over metal particle was 8. It means that each metal particle was surrounded by eight Cy5-avidin conjugates. This number is reasonable when considering that the number of succinimidyl ligand per metal particle is below 20. We also noticed that the released avidin conjugate displayed a 3-fold decrease of emission intensity as compared with the avidin-metal complex in solution, even though the concentration of fluorophore remained

unchanged before and after the treatments, implying that the fluorescence signals by the Cy5-avidin conjugates were 3-fold enhanced by the metal particles [18,19].

The fluorescence images of probes were recorded using scanning confocal microscopy (Figure 1). Because the all fluorophore samples were diluted to nM scale in solution before casting and drying on coverslip, we believed that the most recorded round emission spots came from the single metal-free avidin conjugates or avidin-metal complexes. For each sample, at least 30 emission spots were collected to create the intensity and lifetime profiles (Figure 2). Compared with the metal-free Cy5-avidin conjugates, the avidin-metal complexes were found to display a 10-fold increase of emission intensity and a 2-fold decrease of lifetime [25,26]. Thus, we expect that the avidin-metal complexes have more opportunity to be identified from the cellular autofluorescence of cell lines when they are used to label the cell lines on the surfaces.

PM1 cells were surface-biotinylated using EZ-link sulfo-NHS-biotin [41,42], and then bound with the avidin-metal complexes via biotin-avidin interactions. The cell lines were treated with the different concentrations of biotin reagent, resulting in the different amounts of biotin-site on the cell surfaces. The avidin molecules were in excess amount to ensure the saturation loading of fluorophores on the cell surfaces. The fluorescent cell images were recorded in both the emission intensity and lifetime using scanning confocal microscopy. The fluorescence images of cell samples biotinylated with the different amount of reagents were collected and compared (Figure 3). It was noticed that only the cell lines that were biotinylated could be read out by the confocal microscopy but the untreated cell lines could not, indicating that the existences of biotin sites on the cell surfaces and their interactions with the avidin conjugates play a role in the cell imaging. On the cell images labeled by the metal free avidin conjugates (Figure 3a), no emission spot can be identified clearly; but on the cell images labeled by the avidin-metal complexes (Figure 3b-e), the emission spots can be distinguished from the cellular autofluorescence. The avidin-metal complexes were observed to bind on the cells indicating that these metal particles were not influenced significantly by their steric hindrances when being proximity and binding on the cell surfaces. However, because of the heterogeneity of cell medium on the intensity images, we could not accurately count the number of emission spots on the entire cell images. On the other hand, the emission intensities over the images were observed to change with the concentration of biotin reagent in solution. A population of at least 20 images of cells by the avidin-metal complexes were collected for each sample and the average emission intensities were plotted against the concentration of biotin reagent in solution (Figure 4), showing a significant increase of emission intensity from 50 to 330 with an increase of biotin reagent concentration. Contrarily, the images of cells labeled by metal free avidin conjugates displayed a slight increase with an increase of biotin reagent concentration in solution. For the cell sample treated by the highest concentration of biotin reagent, the emission intensity of cell image by the avidin-metal complexes displayed a 6-fold increase than that by the metal-free avidin conjugates. The reason is the emission signals from the metal probes are much stronger than those from the free avidin conjugates in the absence of metal, which is principally due to MEF. In addition, the coupling interaction from the nearby metal particles on the cell surfaces can also play a role in the enhanced emission. Thus, the avidin-metal complex can be regarded as a better fluorescent probe than the metal-free conjugate in the cell imaging. We noticed that the error bar gets larger with an increase of the number of biotin site on the cell surface in Figures 4. The reason is the increase of avidin-metal complex amount on the cell surface may make it difficult to count closely spaced probes. In addition, the most relative errors are ranged in $\pm 30\%$ indicating that the errors are acceptable in the measurements.

Different from the emission intensity images, the lifetime images of cells were not influenced by the cellular autofluorescence from the heterogeneity of cell medium because the metal probes displayed much shorter much shorter lifetimes. It was shown that although no isolated

emission spot was observed for the cells labeled by the metal-free avidin conjugate, many individual emission spots could be identified distinctly for the cell labeled by the avidin-metal complex (Figure 3). These isolated emission spots were found to distribute densely and homogeneously on the cell images and the emission intensities of them were about 5-fold higher than the surrounding intensity of autofluorescence background. We anticipate the direct counting of emission spots from the 3-D images of cells especially when the cell samples were loaded with a low level of avidin-metal complex and the emission spots from the bound avidin metal complexes could be identified distinctly from the cellular autofluorescence. The z-scanning was used to achieve the 3-D images. The cell images at the different cut-layers were combined into one image using OriginPro 7.0 software [26]. However, the isolation and counting of emission spots on the cell images with a high level of avidin-metal complex became difficult or even impossible because the emission spots were too dense and became continuous when the cell lines were treated by more than 1 nM biotin reagent in solution. This result is reasonable when considering that the resolution of confocal microscopy is only 200 nm but the size of protein molecule is much smaller. Thus, we count the emission spots only from the images of cells biotinylated by below 1 nM in solution. The emission intensities of cell images were plotted against the amounts of emission spots (Figure 5), showing a significant increase with an increase of avidin-metal complexes, which was interpreted by an increase of biotin sites on the cell surfaces. We think that although the biotinylation of cell surface may not be strictly linear with the reagent concentration, we believe that the amount of biotin site on the cell surface should increase with the reagent concentration. In addition, the avidin-metal complex is in excess amount, so the amount of avidin-metal complex should increase with an increase of biotin amount on the cell surface.

Besides the emission intensity, we observed that the lifetime over the entire cell image was significantly altered with the amount of avidin-metal complex on the cell surface. Thus, the lifetime parameter could also be used instead of the emission intensity to create a quantitative regression curve for the target molecule on the cell surfaces. In the treatments, the time-resolved emission intensity decays from the bound avidin-metal complexes on the cell images were analyzed in term of two-component model, and the two components were estimated to be average 2.7 ns (longer component) and 0.8 ns (shorter component), respectively. The shorter component is close to the lifetime of unbound avidin-metal complex and the longer component may be from the cellular autofluorescence of cell line. The histograms of lifetimes over the entire cell image displayed a significant shifting from 1.7 ns for the cell sample with a low level of biotinylation to 1.1 ns for the cell sample with a high level of biotinylation (Figure 6), shortening with an increase of the metal probe amount on the cell surface. It was due to the overlap of fluorescence signals from the bound avidin-metal complexes with short-lifetimes and cellular autofluorescence with longer lifetimes. The lifetime became shorter over the image of cell with more avidin-metal complexes on the surface accompanying with an increase of fractional contribution from the shorter lifetime component on the cell lifetime images. The average lifetimes were plotted against the number of emission spots on the cell images (Figure 5), showing an inverse correlation. Similar to the emission intensity curve, the lifetime curve can also be used to estimate the precise amount of target molecules on the cell surface. Compared with the curve of emission intensity, we prefer the curve of lifetime in the estimation, because the intensity image of cell is observed to be sensitive to the power of excitation laser, but the lifetime is not. Thus, the lifetime curve is believed to be more reliable in the quantification of target molecules on the cell surfaces. By this approach, we can simple and rapidly estimate the number of probe on the cell surface. For instance, extending the curves of emission intensity and lifetime in Figure 5 to 10 nM, we may estimate there are about 150 and 130 emission spots, respectively, on per cell image. By the same way, we also can use this model to infer the precise number of target molecules on the cell surfaces.

The lifetime of cell sample labeled by the metal-free avidin conjugate was 2.2 ns, close to the lifetime of Cy5-avidin conjugate but shorter than the value of cellular autofluorescence [23].

We are also interested in the photostability of fluorophores in the imaging measurements when they are bound on the cell surfaces. The time-trace profiles of emission spots by the avidin-metal complexes were recorded and compared with those by the metal-free Cy5-avidin conjugates. To the metal-free avidin conjugates, because no clear emission spot from the bound Cy5-avidin conjugate could be isolated from the cellular autofluorescence, we just recorded the areas with the high brightness on the cell images as the profiles. It was shown that the profiles of cell lines labeled by either metal free conjugates or avidin-metal complexes displayed the direct intensity decays with the irradiation time (Figure 7), corresponding to the photobleaching of the fluorophores. However, the decay speeds were different in the two profiles: the metal-free avidin conjugates were completely eliminated within 30 seconds but the avidin-metal complexes were reduced only slightly within 60 seconds. This fact indicates that the avidin-metal complexes on the cell surfaces are more photostable than the metal free avidin conjugates. These avidin-metal complexes were observed to be eliminated completely in 600 seconds, 10-fold longer than the metal-free avidin. Because only the isolated emission spots were monitored in the time-trace measurements, we believe that the photostability of bound fluorophores on the cell surfaces were solely impacted by the presence of metal but independent of the number of biotinylation on the cell surfaces in this case.

Conclusion

In this study, Cy5-avidin conjugates were covalently bound on the 20 nm silver particles to work as the molecular imaging reagents. PM1 cell lines were surface-biotinylated by EZ-link sulfo-NHS-biotin and then fluorescently labeled by the avidin-metal complexes. The cell images were recorded using scanning confocal microscopy. It was shown from the cell images that the avidin-metal complexes were bound well on the cell surfaces showing no obvious influence from the steric hindrance of metal particle. Relative to the metal-free avidin conjugate, the avidin-metal complex bound on the cell surface displayed stronger emission signal, shorter lifetime, and better photostability. Thus, when the cell surfaces were biotinylated at a low level, the emission spots from the bound avidin-metal complexes on the cell surfaces could be distinguished from the cellular autofluorescence of cell line and became countable. In addition, the lifetime over the entire cell images was significantly altered with the amount of biotin-site on the cell surfaces. A quantitative regression curve could be achieved deal with the correlation between the amount of avidin-metal complex on the cell surface and the emission intensity or lifetime maxima over the entire cell image. This approach can be used to estimate the precise amount of target molecule on the cell surface at the single cell level.

Acknowledgements

This research was supported by grants from NIH (HG-00255, EB006521, and EB00682 to JRL) and research support from the University of Maryland Medical Center (RZ).

References

1. Lakowicz, JR. Principles of Fluorescence Spectroscopy. Vol. 3rd edition. Kluwer Academic / Plenum Publishers; New York: 2006.
2. Mason, W., editor. A Practical Guide to Technology for Quantitative Real-Time Analysis: Fluorescent and Luminescent Probes for Biological Activity. Academic Press; 1999.
3. Ambrose WP, Goodwin PM, Jett JH, Van Orden A, Werner JH, Keller RA. Chem. Rev 1999;99:2929. [PubMed: 11749506]
4. Willets KA, Nishimura SY, Schuck PJ, Twieg RJ, Moerner WE. Acc. Chem. Res 2005;38:549. [PubMed: 16028889]

5. Fu A, Gu W, Boussert B, Koski K, Gerion D, Manna L, Le Gros M, Larabell CA, Alivisatos AP. *Nano Lett* 2007;7:179. [PubMed: 17212460]
6. (a) Thoumine O, Ewers H, Heine M, Groc L, Frischknecht R, Giannone G, Poujol C, Legros P, Lounis B, Cognet L, Choquet D. *Chem. Rev* 2008;108:1565. [PubMed: 18447398] (b) Shangguan D, Meng L, Cao ZC, Xiao Z, Fang X, Li Y, Cardona D, Witek RP, Liu C, Tan W. *Anal. Chem* 2008;80:721. [PubMed: 18177018]
7. Rosenthal SJ, Tomlinson I, Adkins EM, Schroeter S, Adams S, Swafford L, McBride J, Wang Y, DeFelice LJ, Blakely RD. *J. Am. Chem. Soc* 2002;124:4586. [PubMed: 11971705]
8. Chen I, Ting AY. *Current Opinion in Biotech* 2005;16:35.
9. Weissleder R, Tung CH, Mahmood U, Bogdanov A. *Nature Biotechnol* 1999;17:375. [PubMed: 10207887]
10. Ntziachristos V, Chance B. *Breast Cancer Res* 2001;3:41. [PubMed: 11250744]
11. Rosi NL, Mirkin CA. *Chem. Rev* 2005;105:1547. [PubMed: 15826019]
12. Santra S, Zhang P, Wang K, Tapeç R, Tan W. *Anal. Chem* 2001;73:4988. [PubMed: 11681477]
13. Knemeyer J-P, Herten D-P, Sauer M. *Anal. Chem* 2003;75:2147. [PubMed: 12720354]
14. (a) Sokolov K, Chumanov G, Cotton TM. *Anal. Chem* 1998;70:3898. [PubMed: 9751028] (b) Chumanov G, Sokolov K, Gregory BW, Cotton TM. *J. Phys. Chem* 1995;99:9466.
15. (a) Shen Y, Swiatkiewicz J, Lin T-C, Markowicz P, Prasad PN. *J. Phys. Chem. B* 2002;106:4040. (b) Stoermer RL, Keating CD. *J. Am. Chem. Soc* 2006;128:13243. [PubMed: 17017805] (c) Nicewarner-Pena SR, Carado AJ, Shale KE, Keating CD. *J. Phys. Chem. B* 2003;107:7360.
16. (a) Chen Y, Munechika K, Ginger DS. *Nano Lett* 2007;7:690. [PubMed: 17315937] (b) Xie F, Baker MS, Goldys EM. *Chem. Mater.* 2008ASAP Article
17. (a) Yu F, Persson B, Lofas S, Knoll W. *J. Am. Chem. Soc* 2004;126:8902. [PubMed: 15264814] (b) Ekgasit S, Thammacharoen C, Yu F, Knoll W. *Anal. Chem* 2004;76:2210. [PubMed: 15080730] (c) Balushev S, Yu F, Miteva T, Ahl S, Yasuda A, Nelles G, Knoll W, Wegner G. *Nano Lett* 2005;5:2482. [PubMed: 16351199]
18. Lakowicz JR. *Anal. Biochem* 2005;337:171. [PubMed: 15691498]
19. Lakowicz JR. *Anal. Biochem* 2001;298:1. [PubMed: 11673890]
20. Ghosh SK, Pal T. *Chem. Rev* 2007;107:4797. [PubMed: 17999554]
21. (a) Mertens H, Koenderink AF, Polman A. *Phys. Rev. B* 2007;76:115123. (b) Kumbhar AS, Kinnan MK, Chumanov G. *J. Am. Chem. Soc* 2005;127:12444. [PubMed: 16144364] (c) Bruzzone S, Malvaldi M, Arrighini GP, Guidotti C. *J. Phys. Chem. B* 2005;109:3807. [PubMed: 16851429]
22. (a) Zhang J, Fu Y, Chowdhury MH, Lakowicz JR. *J. Phys. Chem. C* 2008;112:18. (b) Zhang J, Fu Y, Chowdhury MH, Lakowicz JR. *Nano Lett* 2007;7:2101. [PubMed: 17580926]
23. Fu Y, Zhang J, Lakowicz JR. *Langmuir* 2008;24:3429. [PubMed: 18278953] (b) Ray K, Badugu R, Lakowicz JR. *J. Am. Chem. Soc* 2006;128:8998. [PubMed: 16834349]
24. Stewart ME, Anderton CR, Thompson LB, Maria J, Gray SK, Rogers JA, Nuzzo RG. *Chem. Rev* 2008;108:494. [PubMed: 18229956]
25. Zhang J, Fu Y, Lakowicz JR. *Bioconj. Chem* 2007;18:800.
26. Zhang J, Fu Y, Liang D, Nowaczyk K, Zhao RY, Lakowicz JR. *Nano Lett* 2008;8:1179. [PubMed: 18341300]
27. Saalik P, Elmquist A, Hansen M, Padari K, Saar K, Viht K, Langel U, Pooga M. *Bioconjugate Chem* 2004;15:1246.
28. Link AJ, Vink MKS, Tirrell DA. *J. Am. Chem. Soc* 2004;126:10598. [PubMed: 15327317]
29. Dontha N, Nowall WB, Kuhr WG. *Anal. Chem* 1997;69:2619. [PubMed: 9230677]
30. Smiley RD, Hammes GG. *Chem. Rev* 2006;106:3.
31. Kreibig, U.; Vollmer, M. *Optical Properties of Metal Clusters*, Springer Series in Materials Science. Vol. 25. Springer-Verlag: Berlin and Heidelberg; Germany: 1995. Hayat, MA., editor. *Colloidal Gold: Principles, Methods, and Applications*. Academic Press; San Diego: 1991.
32. (a) Zhang J, Roll D, Geddes CD, Lakowicz JR. *J. Phys. Chem. B* 2004;108:12210. (b) Huang T, Murray RW. *Langmuir* 2002;18:7077.

33. (a) Templeton AC, Wuelfing WP, Murray RW. *Acc. Chem. Res* 2000;33:27. [PubMed: 10639073]
(b) Ingram RS, Hostetler MJ, Murray RW. *J. Am. Chem. Soc* 1997;119:9175.
34. Heilemann M, Herten DP, Heintzmann R, Cremer C, Muller C, Tinnefeld P, Weston KD, Wolfrum J, Sauer M. *Anal. Chem* 2002;74:3511. [PubMed: 12139062]
35. Osborne MA, Barnes CL, Balasubramanian S, Klenerman D. *J. Phys. Chem. B* 2001;105:3120.
36. Frangioni JV. *Curr. Opin. Chem. Biol* 2003;7:626. [PubMed: 14580568]
37. (a) Osella D, Pollone P, Ravera M, Salmain M, Jaouen G. *Bioconj. Chem* 1999;10:607–612. (b) Kinter AL, Poli G, Fox L, Hardy E, Fauci AS. *J. Immunol* 1995;154:2448. [PubMed: 7868911]
38. Wersinger C, Vernier P, Sidhu A. *Biochemistry* 2004;43:1242. [PubMed: 14756560]
39. Wersinger C, Sidhu A. *Biochemistry* 2005;44:13612. [PubMed: 16216085]
40. Zhang J, Malicka J, Gryczynski I, Leonenko Z, Lakowicz JR. *J. Phys. Chem. B* 2005;109:7643. [PubMed: 16851886]
41. Yuan F, Dellian M, Fukumura D, Leunig M, Berk DA, Torchilin VP, Jain RK. *Cancer Res* 1995;55:3752. [PubMed: 7641188]
42. Wojda U, Goldsmith P, Miller JL. *Bioconjugate Chem* 1999;10:1044. (b) Chang PV, Prescher JA, Hangauer MJ, Bertozzi CR. *J. Am. Chem. Soc* 2007;129:8400. [PubMed: 17579403]
43. (a) Fu Y, Lakowicz JR. *Anal. Chem* 2006;78:6238. [PubMed: 16944907] (b) Fu Y, Lakowicz JR. *J. Phys. Chem. B* 2006;110:22557. [PubMed: 17092001]
44. Hu J, Zhang J, Liu F, Kittredge K, Whitesell JK, Fox MA. *J. Am. Chem. Soc* 2001;123:1464.

Figure 1. Representative lifetime images of (a) single metal-free Cy5-avidin molecules and (b) single avidin-metal complexes. The images were acquired by scanning confocal microscopy. The scales of diagrams are $5 \times 5 \mu\text{m}$. The resolution is 200×200 pixel with an integration of 0.6 ms/pixel.

Figure 2. Histograms of emission intensity and lifetime of metal-free avidin and avidin-metal complex counted from their fluorescence images.

Lifetime Images

Intensity Images

Figure 3.

(a) Representative emission intensity and lifetime images of PM1 cells labeled by (a) metal-free avidin or (b-d) avidin-metal complex treated by different concentrations of biotin reagent in solution. The (a) cell sample was treated at 1 nM of biotin reagent and then labeled by 1 μ M metal free Cy5-avidin conjugate. The (b)-(e) cell samples were treated at (b) 10^{-2} nM, (c) 10^{-1} nM, (d) 1 nM, and (e) 10 nM of biotin reagent and then labeled by 0.1 μ M avidin-metal complex. The scales of diagrams are $20 \times 20 \mu\text{m}$. The resolution is 400×400 pixel with an integration of 0.6 ms/pixel.

Figure 4.

Dependences of the emission intensity acquired from the intensity images of cell lines that were bound by the metal-free Cy5-avidin conjugate and avidin-metal complex, respectively on the concentration of biotin reagent in solution.

Figure 5.

Dependence of the emission intensity and lifetime over the cell images on the counting number of emission spot acquired from the lifetime images.

Figure 6.

Lifetime histograms acquired from the overall lifetime images of PM1 cells labeled by the either metal-free avidin or avidin-metal complex. The (a) cell sample was treated at 1 nM of biotin reagent and then labeled by 1 μ M metal free Cy5-avidin conjugate. The (b)-(e) cell samples were treated at (b) 10^{-2} nM, (c) 10^{-1} nM, (d) 1 nM, and (e) 10 nM of biotin reagent and then labeled by 0.1 μ M avidin-metal complex.

Figure 7. Comparison of the time traces acquired from the metal-free Cy5-avidin conjugate and avidin-metal complex that were bound on the surfaces of cell lines, respectively.

Scheme 1.

Tiopronin-coated silver particles are succinimidylated by succinimidyl ester-terminated thiolate compound via ligand exchange, covalently bound by Cy5-avidin conjugates via condensation, and bound on the biotinylated surface of cell line via biotin-avidin interaction. The small red dots represent Cy5-avidin conjugates. The large red dots represent the silver particles coated with Cy5-avidin conjugates. The grey dots represent biotin sites on the cell surfaces.