Fluorescent Clusters

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Shuttle-Based Fluorogenic Silver-Cluster Biolabels**

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Most molecular or cellular labeling utilizes organic dyes that are conjugated to bioactive molecules by using general organic or inorganic reactions. Although the shortcomings of organic dyes such as poor photostability[1] and low brightness[2] limit the observable copy numbers, their lack of selectivity in labeling often more seriously limits their applications in live-cell imaging and single-molecule studies.[3] Specificity can be addressed through antibodies and other strong-affinity pairs such as avidin–biotin,[4,5] but direct covalent technologies are crucial at subpicomolar concentrations, as this is beyond the binding limits of antibody-based affinities. Genetically encoded fluorescent proteins are an excellent solution for specific fluorescent labeling in live cells, but the disadvantageous photophysical instabilities of organic dyes (blinking and bleaching) remain, coupled with the potential perturbation because of large label sizes.[6] Attempts to overcome photoinstabilities of organic labels have produced much brighter quantum-dot labels,[7] but, while providing excellent signals, these emitters introduce additional problems such as large physical size, aggregation, toxicity, polyvalency, and strong fluorescence intermittency.[8–10] Newly emerging silver-nanocluster-based labels, which simultaneously address concerns of brightness, photostability, monovalency, size, and fluorescence intermittency, offer excellent potential as molecular labeling agents.[11–15] Spectrally pure emitters have been produced that have an emission range from the blue to the near-infrared, with fluorescence quantum yields (Φ_F) of up to 40% and hydrodynamic radii of the fully assembled ssDNA-encapsulated silver nanoclusters (ssDNA = single-stranded DNA) of approximately 2.5 nm.[16] Moreover, at both the bulk and single-molecule levels, silver nanoclusters also show both excellent brightness and photostability.[17]

Silver nanoclusters (that is, silver clusters or SCs) are typically created within ssDNA scaffolds through direct reduction of Ag salts by BH_4^- ions in the presence of DNA.[12] Such methods can be employed to generate highly emissive labels on proteins of interest, but the BH_4^- ion is a rather harsh reductant which may reduce disulfide bonds and potentially adversely affect protein function. Consequently, we investigated other scaffolds in which clusters could first be synthesized, but would enable whole cluster transfer to the ssDNA encapsulant—a fluorogenic cluster shuttle. Poly(acrylic acid) (PA), which has been identified to stabilize SC emission,[18] was investigated as a low-quantum-yield silver-cluster scaffold. Initial attempts to create SCs within a commercially available, small linear PA by reduction with sodium borohydride yielded both very low SC concentrations and low Φ_F values, with the simultaneous generation of silver nanoparticles. The formation of nanoparticles was eliminated and the yield of clusters was greatly improved when silver ions were first complexed with 3-(2-aminoethylamino)propyltrimethoxy silane (APTMOS) before PA stabilization and borohydride reduction. Mass spectra of the complexes predominantly show 2:1 APTMOS/Ag^+ complexes, which are likely to limit the final size of the Ag clusters (see the Supporting Information). Ag clusters made in this way (PA–SCs) exhibit a Φ_F value of 3% and a biexponential fluorescence decay (280 ps (27%) and 1380 ps (73%)).

Although their preparation may be easily scaled up to produce large amounts of clusters (that is, more than 100 mL of solution, see the inset in Figure 1a) and they are quite stable for weeks, PA–SCs readily transfer SCs to high-affinity ssDNA sequences, which results in the loss of PA–SC fluorescence and generation of characteristic DNA-encapsulated SC emission. Concomitant with the spectral shifts, cluster transfer from low-quantum-yield PA–SC to high-quantum-yield ssDNA–SC results in a 10-fold increase in cluster brightness, with excellent specificity. Immediately upon mixing PA–SC with 12-mer oligocytosine (C12), the emission intensity increases significantly (about ten times), and the excitation spectra shift correspondingly from that of PA–SC ($\lambda_{max} = 515$ nm) to that of 12-mer polycytosine (C12–SCs, $\lambda_{max} = 570$ nm, Figure 1a). The transfer efficiency depends on the composition of PA, Ag^+, and APTMOS (see the Supporting Information), with each Ag^+/PA ratio having an optimal ratio of Ag^+/APTMOS. At lower Ag^+/PA ratios, a lower Ag^+/APTMOS ratio yields more efficient SC transfer, and vice versa. C12–SCs exhibit reasonably high initial count rates after SC transfer with Ag^+/PA and Ag^+/APTMOS ratios of 3, as used in this work. The emission intensity continues to increase, and reaches the emission intensity of directly prepared C12–SCs within 30 minutes. The pH of the solution and buffer conditions also influence SC transfer, with cluster transfer being further facilitated at a higher pH value, presumably through destabilization of the PA–SC complex. While working well for multiple 12-mer sequences, precise transfer conditions may need to be optimized for specific sequences that exhibit secondary structures or intermolecular interactions. For example, cluster transfer to 5′-

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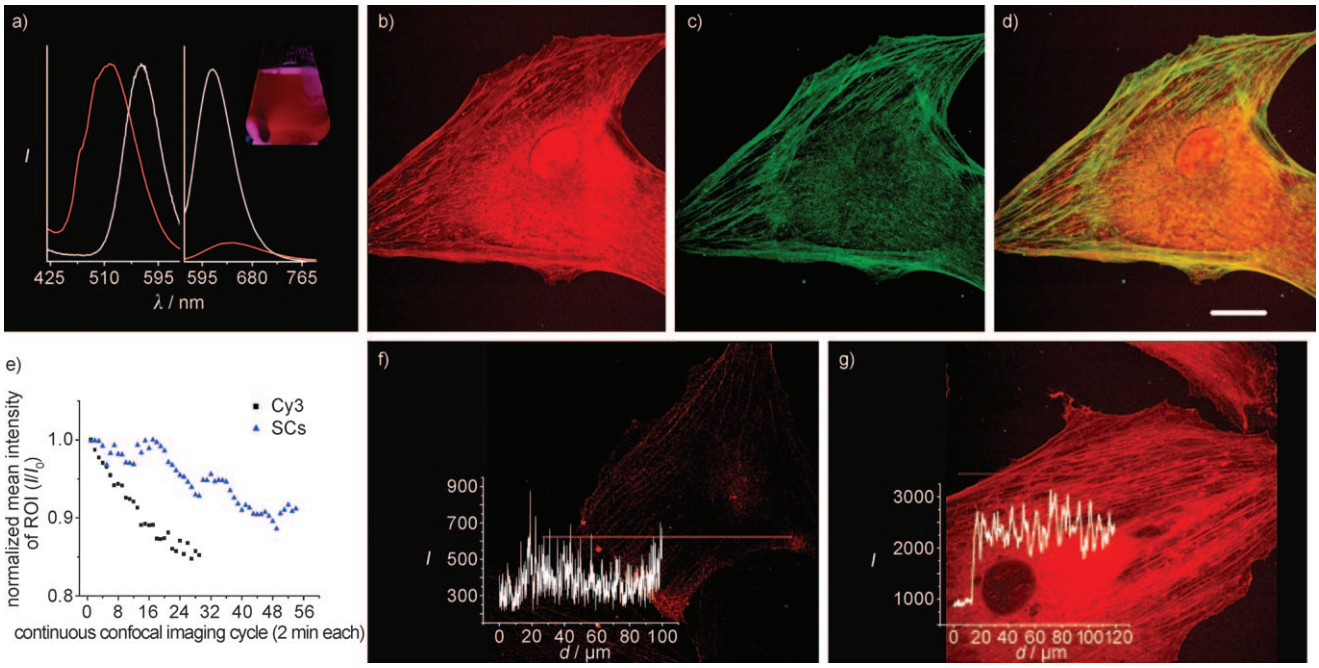

Figure 1. BPAECs stained with anti-actin Ab/C12 (actinC12) and visualized by SC transfer. a) SCs transferred from PA to C12. Left panel, normalized excitation spectra of reaction mixture detected at 640 nm before (red) and after (white) the addition of C12, which corresponds to individual excitation spectra of PA–SCs and C12–SCs, respectively. Right panel, 10-fold increase in the fluorescence intensity after the addition of C12 (white); excited at 545 nm. The inset shows PA–SCs (100 mL) prepared in a Pyrex conical flask, under 354 nm excitation. b) Fluorescence images of BPAEC from SCs and c) FITC tagged to actinC12 to indicate the location of actin, with colocalization in (d). e) Photostability and f, g) brightness comparison between Cy3-labeled (f) and SC-labeled (g) cells under identical staining and imaging conditions. The insets show the intensity profiles versus distance *d* along the indicated line within the selected images. SCs and Cy3 were excited at 543 nm (20 Wcm^{-2}); FITC was excited at 488 nm. The scale bar is 25 μm.

AATTCCCCCCCCCCCCAATT-3′ (ATC12) does not proceed at room temperature, but occurs readily at 50 °C. This moderate selectivity allows SC transfer to ssDNA-tagged proteins with little interference from other cellular components. Generally, once protein fluorescence is desired, SCs can be transferred to the ssDNA–protein conjugate. For an ordinary antibody, the DNA tag is much more easily handled than light-sensitive dyes. Most importantly, the brightness and photostability advantages of SCs can be utilized for higher-sensitivity imaging.

Using our fluorogenic SC shuttle, we fluorescently labeled cellular components by first conjugating C12 to antibodies (Abs), staining with these nonfluorescent DNA-conjugated antibodies, and transferring SCs as the final step. Actin, one of the three major cytoskeleton components,[19,20] was stained with the anti-actin Ab/C12 conjugate (actinC12) prepared from the anti-actin Ab/FITC conjugate (Sigma, Saint Louis, Missouri, USA) and C12 by linking with sulfo-SMCC (4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulfo-*N*-hydroxysuccinimide ester sodium salt). The efficiency was examined in methanol-fixed, acetone-permeated BPAECs (bovine pulmonary artery endothelial cells), which were first stained with actinC12 (1:50 dilution) overnight at 4 °C or 1 hour at room temperature, and subsequently incubated with PA–SCs for 20 minutes. The emission image from the SCs (Figure 1 b) colocalized well with that of the FITC (Figure 1 c) that was tagged to the anti-actin Ab, thus revealing antibody location and indicating actin staining with SCs by SC transfer (Figure 1 d). Controls showed that long-term staining with PA–SCs alone (without antibody staining) yielded no such emission images and there was no silver-cluster transfer between PA–SCs and the antibody unless the ssDNA label was also present. The images show typical actin morphology, which implies that conjugation to DNA does not interfere with antibody function. The conjugate, however, produces some nonspecific nuclear staining that is readily reduced by blocking with BSA (Figure 1 g).

The brightness of BPAECs stained with either anti-actin Ab/Cy3 dye conjugate or actinC12 and subsequent SC transfer as the last step was compared over many preparations under identical labeling and imaging conditions. The images from the SCs are generally about seven times brighter than those employing the Cy3 dye (Figure 1 f, g). Since the Cy3/Ab ratio is 8, and the C12/Ab ratio is 3, each SC is approximately 20 times brighter than each Cy3 dye unit under these conditions, given that the same type of antibody staining of the same batch of cells should result in quite similar staining. In addition to excellent brightness, SCs also exhibit outstanding photostability. Over two hours of slow scanning on a Zeiss confocal microscope, the SC-stained BPAECs showed only a 10 % decrease in emission intensity, which is significantly better than that of Cy3 (Figure 1 e), while simultaneously being much brighter overall. This silver cluster transfer protocol also works well on other cell lines, such as NIH 3T3 (see the Supporting Information). The availability of SC transfer, together with the outstanding brightness and photostability of SCs further illustrates their promise as bulk and single-molecule labels.

The anti-α-tubulin/C12 conjugate (tubulinC12, Invitrogen) was similarly synthesized to stain microtubules.[21] In this case, SCs were transferred to tubulinC12 (primary Ab), which had been immunologically targeted by an Alexa-488-tagged secondary antibody (Alexa Fluor 488 goat anti-mouse IgG, Invitrogen). Good colocalization of the emission image of BPAECs from SCs and Alexa 488 (see the Supporting Information) indicates that, again, the transfer is quite efficient even though the secondary Ab may partially block the access of PA–SCs to tubulinC12. The small size of PA–SCs may contribute to this significant silver-cluster permeability.

The transfer fidelity, coupled with the excellent brightness and photostability of SCs encouraged us to further apply SC transfer to live-cell labeling. We utilized antifibroblast surface protein antibodies (anti-FSP) to recognize an epitope on live fibroblast cells.[22] The synthesis of anti-FSP 24-mer polycytosine conjugate (FSPC24) is also straightforward, with sulfo-SMCC as a linker between C24 and anti-FSP (Sigma). The conjugate was then loaded into the growth medium of NIH 3T3 cells at 4 °C for 15 minutes, followed by SC transfer from PA–SCs at 4 °C for 10 minutes. At the recommended 1:500 dilution of FSPC24, the cell surface showed uneven staining after SC transfer (data not shown) because of short incubation time, low temperature, and low antibody concentration. To improve staining, the Ab concentration was increased 30-fold and the incubation time was decreased to avoid the characteristic cellular detachment resulting from the propensity of anti-FSP to eliminate fibroblast cells from a mixture of varied cell lines.[23] Under these conditions, much brighter and more uniform staining resulted (Figure 2a). Reconstruction of z-stack confocal images also shows clear cell-surface staining (Figure 2b). Cells incubated with C24-encapsulated silver clusters alone, either at 4 °C or 37 °C, showed no staining from silver clusters, which suggested antibody-mediated staining. While quite promising as a fluorogenic cluster shuttle, only surface proteins can currently be labeled for live cells because of the poor cell permeability of PA–SCs. However, SC transfer can be useful in the study of receptor-mediated target delivery. As shown in Figure 2c, when the target FSPC24 staining cell surface protein was marked with silver clusters by SC transfer at 4 °C, and then incubated at 37 °C for 30 minutes, FSPC24 silver clusters were internalized, quite likely by endocytosis, which resulted in punctuate staining inside the cells. Nevertheless, coupled with both DNA-sequence-directed SC emission colors[16] and specific peptide scaffolds for silver-cluster binding[24] the design of new shuttles may enable direct multicolor intracellular labeling in future studies.

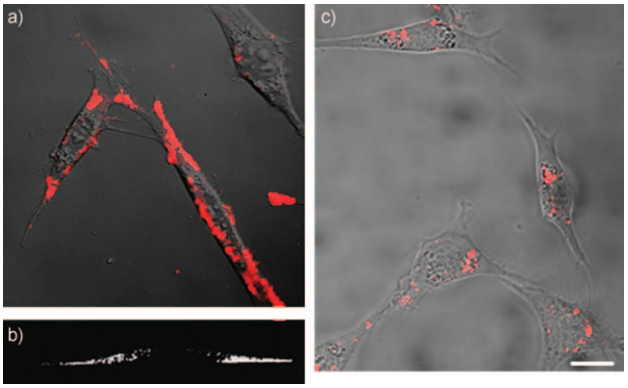

Figure 2. Live NIH 3T3 cells stained with FSPC24 with SC transfer as the last step. a) Merge brightfield and SC fluorescence (pseudo color red, excited at 543 nm). Cells were incubated with FSPC24 at 4 °C. b) Orthoaxis slice of the long cell in the lower-right region of (a), reconstructed from z stacks of confocal images, showing cell surface staining at 4 °C. Scale bar, 30 μm. Because of the low labeling employed to maintain adherence, thicker slices are used, which artificially suggests less labeling of the top of the cell. c) Merge brightfield and SC fluorescence of cells as in (a), with cells incubated at 37 °C.

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