Cell Staining

DOI: 10.1002/anie.200604253

In Vitro and Intracellular Production of Peptide-Encapsulated Fluorescent Silver Nanoclusters**

*Junhua Yu, Sandeep A. Patel, and Robert M. Dickson**

Recently developed labeling technologies have enabled new insights into cellular function and biomolecular interaction. While great advances have been made in encodable probes,[1] molecular labels,[2] ultrabright emitters,[3] and specific coupling chemistries,[4] the benefits of a single combined technology offering small size, bright emission, and potential genetic encodability has yet to be realized. Within the past few years, low-nuclearity silver nanoclusters have offered promise to combine small size and potentially ultrabright emission in a variety of scaffolds, which stabilize silver nanocluster emission ranging in brightness from that of typical organic dyes[5] to beyond that of semiconductor quantum dots.[6,7] On the basis of a nucleolus-bound silver-binding protein, we report a significant advance toward utilizing silver nanocluster emission within living cells and the potential for further optimization of small peptide scaffolds for improved nanocluster optical properties.

Frequently used by pathologists, silver nanoparticle-based staining has been utilized to detect nucleolus organizing regions within cells and thus partially reveal nucleolus location.[8,9] This method is thought to reflect cell proliferation activity of potentially cancerous cells.[10] The rather harsh, general protocol for nucleolar silver staining within fixed cells (>5.8 M silver nitrate, 60 °C) yields large metallic silver nanoparticles that appear as dark spots under bright-field microscopy or electron microscopy as a result of the interaction between argyrophilic proteins and silver.[11] Considering the specific nucleolar staining with large silver nanoparticles, we expected that fluorescent silver nanoclusters could be produced in vivo in the nucleoli of cells by ambient-temperature photoactivation, and that one of the known silver-binding proteins, nucleolin, may serve as a guide for templating the high efficiency creation of fluorescent silver nanoclusters.

Although encountering problems with alteration of activity and autofluorescence, especially at low copy numbers, live-cell imaging studies often employ green fluorescent protein fusions, expensive fluorescent antibodies, or specifically designed dyes against certain subcellular components for visualization with fluorescence microscopy.[12] Circumventing background emission, novel long-lived europium complexes designed for nucleolar targeting can be temporally distinguished using time-gated microscopy.[13] While such long-lived emitters increase the bulk signal/noise (S/N) ratio, their low emission rates preclude observation of low copy numbers. Recent instrumental developments now enable imaging of very short lifetime materials as well.[14] Such fast (ca. 100 ps) gating could enable fast emitters to be observed before standard dyes (nanoseconds) begin to emit. When combined with silver nanoclusters exhibiting picosecond lifetimes,[6,15] high S/N imaging of noble metal nanoclusters should be readily accomplished even in the presence of other dyes.

Contrary to the ordinary harsh silver staining conditions, fluorescent silver nanocluster formation can be initiated and used to stain the cells at much lower $AgNO_3$ concentrations (20 mM, Supporting Information) by photoactivation at ambient temperature. Such images (Figure 1) always emphasize several bright regions reminiscent of nucleoli, on top of

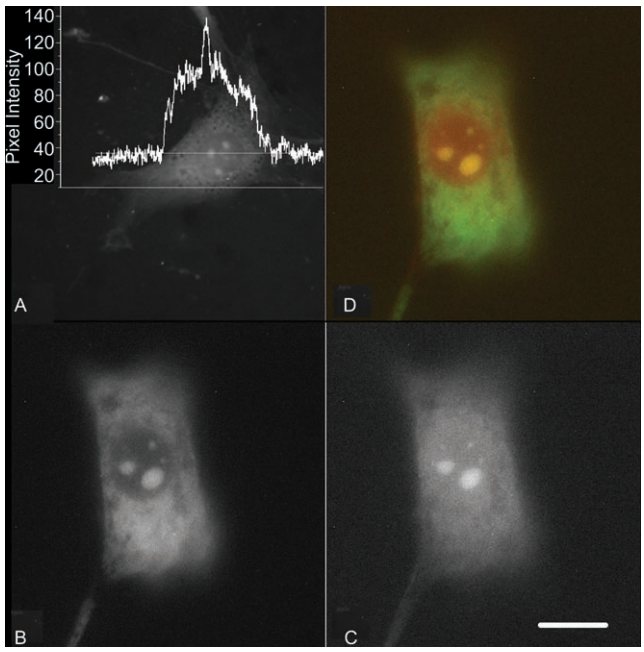

Figure 1. Emission from formaldehyde-fixed NIH 3T3 cells loaded with 100 mM silver nitrate for 20 h. A) Fluorescence image; the inset is the intensity profile along the line drawn across the cell. B)–D) Colocalization of silver and SYTO RNASelect staining; B) emission from RNASelect fluorescence (green channel); C) emission from silver clusters (red channel); D) merge of (B) and (C). Scale bar: 30 μm.

[*] Dr. J. Yu, S. A. Patel, Prof. R. M. Dickson
School of Chemistry and Biochemistry and
Parker H. Petit Institute for Bioengineering and Bioscience
Georgia Institute of Technology
901 Atlantic Drive, Atlanta, GA 30332-0400 (USA)
Fax: (+1) 404-894-7452
E-mail: dickson@chemistry.gatech.edu

[**] The authors gratefully acknowledge financial support from NSF BES-0323453, Invitrogen Corp., NIH R01M68732, and NIH P20072021, and thank Prof. D. F. Doyle for use of his cell-culture facility, and Prof. Y.-L. Tzeng for the gift of the peptides.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

weak cytoplasmic and nuclear emission. The emission intensity of the "bright regions" is about twice the intensity of that from the cytoplasm, and exhibits broad spectra between 500–700 nm under blue excitation (Supporting Information), likely resulting from multiple emissive species of 2–7 Ag atoms in the cells. The spectra differ somewhat among different cellular components, with the bright spots exhibiting slightly higher ratios of red to green emission than cytoplasm. The emission of the silver-stained cells is quite fast (220 ps (33 %) and 1760 ps (67 %)) and very stable, maintaining more than 50 % intensity for 25 min of continuous illumination at 1 $W cm^{-2}$ (Supporting Information). To confirm fluorescent silver nucleolar staining, colocalization experiments were performed with SYTO RNASelect green fluorescence stain (Invitrogen). As shown in Figure 1, the merged image (D) of RNASelect fluorescence (B, band pass 515–525 nm; green channel) and fluorescent nanocluster silver staining (C, long pass 665 nm; red channel) confirms nucleolar staining.

As the silver clusters exhibit short lifetimes, the silver-stained cells can be monitored with picosecond-gated microscopy to minimize contributions from autofluorescence or other coloaded dyes (Figure 2). The 212-ps IRF-broadened emission centered at the nucleus increases to its maximum intensity within 320 ps and then fades to the autofluorescence level within a further 1500 ps. The variation in emission intensity of cellular images is in line with the lifetime decay of the silver clusters in cells. Not only is this the first report of silver fluorescence of cells, but to our knowledge, is also the first report collecting short-time emission to increase S/N by temporally rejecting slower emission from other coloaded dyes or autofluorescence.

Consisting of more than 700 amino acids, nucleolin was identified as one of the major proteins to bind silver atoms in silver staining,[16] and appears to nucleate the formation of fluorescent silver nanoclusters in our studies. Unfortunately, nucleolin is far too large to be useful as a label. Ligands which can protect the silver clusters are indispensable as the silver clusters are very vulnerable, with reduction potential decreasing from 0.799 V of the conventional silver electrode to −1.8 V of free silver atom.[17] This observation implies that the newly formed silver clusters will be oxidized quickly if no protection is available. As the silver binding site is unknown, we designed a short peptide, KECDKKECDKKECDK (**P1**), incorporating the specific amino acids most prevalent in nucleolin: glutamic acid (15.6 %), lysine (12.7 %), and aspartic acid (8.5 %). We also incorporated several cystine groups based on the known strong silver affinity of thionein.[18] This nucleolin-inspired peptide forms and stabilizes fluorescent silver clusters directly in phosphate buffered saline (PBS), indicating a strong interaction between silver and ligand. However, the silver clusters protected with **P1** (**P1-Ag**) in aqueous solution are only moderately stable at room temperature (chemical lifetime of 3 days). The short peptide length may limit nanocluster stability owing to greater vulnerability to oxygen and chloride ions in aqueous solution. In separate ongoing studies of silver nanocluster creation in specifically designed dendrimers, we observed that hydrophobic regions facilitate the formation of silver clusters, leading us to improve the peptide by incorporating several hydrophobic amino acids to yield HDC-HLHLHDCHLHLHCDH (**P2**) and HDCNKDKHDCNKDKH-DCN (**P3**). Silver clusters protected with **P2** and **P3** (**P2-Ag** and **P3-Ag**) are much more stable (chemical lifetime of 2 weeks in deionized water and 5 weeks in PBS, respectively). Nevertheless, all the peptide-protected silver clusters are stable at −20 °C for at least one month.

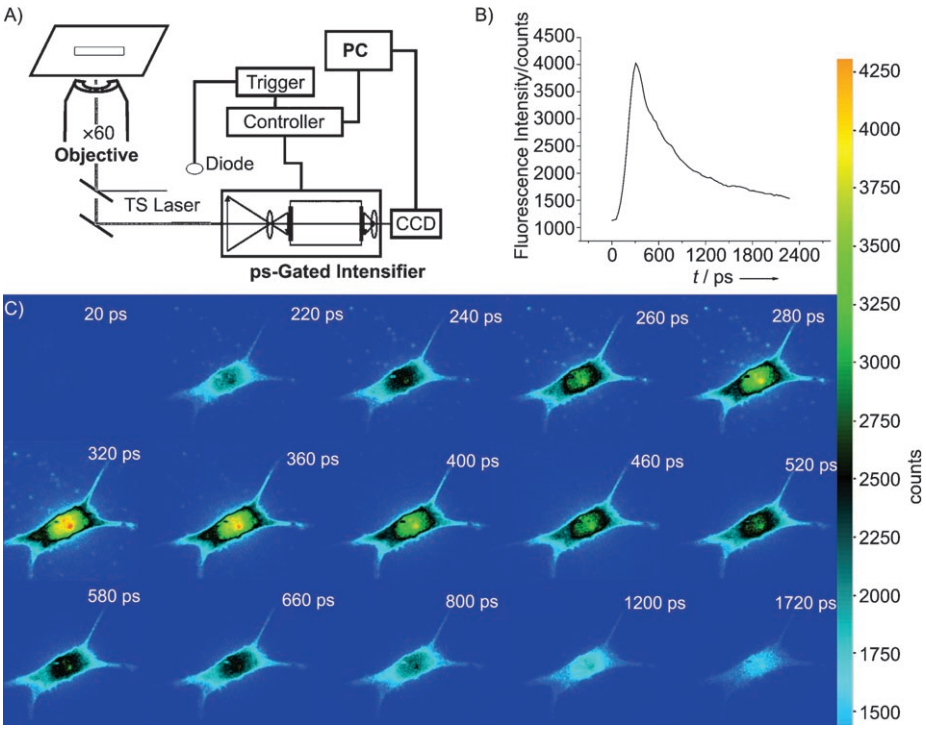

Figure 2. Time-gated images of NIH 3T3 cells stained with silver nitrate. A) Microscope setup integrated with ps-gated intensifier (LaVision Picostar HR) and CCD camera (Andor iXon). B) Time slices of a cell stained with silver nitrate. C) Time profile of the time series images showing the fast silver nanocluster emission at short times. Note that black indicates an intermediate intensity level in this color scheme.

These silver clusters exhibit similar photophysics to those in the nucleolus, but with narrower emissions at 610 nm (**P1-Ag**), 615 nm (**P2-Ag**), and 630 nm (**P3-Ag**) (Figure 3 A and Supporting Information). The MALDI mass spectrometry confirms the presence of silver clusters, in which Ag_2, Ag_3, Ag_4, or Ag_5 are bound to a single peptide (Figure 3 B). The fluorescence decay of **P1-Ag** can be fit biexponentially, with a fast component of 260 ps (58 %) and a slow component of 2300 ps (42 %). Silver clusters prepared in **P2** and

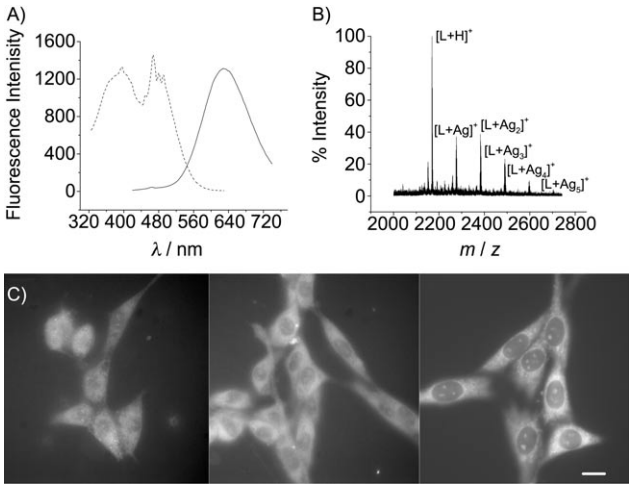

Figure 3. Photophysics of **P3-Ag** and its cell loading. A) Emission and excitation spectra of aqueous **P3-Ag** solution. Emission (solid trace) was excited at 400 nm, and excitation (dotted trace) was detected at 630 nm. B) MALDI mass spectrum of **P3-Ag**. C) Fluorescence images of NIH 3T3 cells loaded with **P3-Ag** (0.5 mg): left: in DMEM at 37 °C and 5% CO_2; center: in DMEM at 4 °C; right: methanol-fixed NIH 3T3 cells loaded with **P3-Ag** in DMEM at room temperature (incubation time: 1 h). Under these imaging conditions, no detectable emission from the control cells without Ag nanoclusters is observed (not shown). Scale bar: 30 μm.

P3 also exhibit similar lifetimes (**P2-Ag**: 73 ps (15%) and 1900 ps (85%); **P3-Ag**: 420 ps (36%) and 2900 ps (64%)). The photophysical similarity between silver-stained cells and peptide-protected silver clusters also strongly supports the observation of fluorescent silver clusters in cells. This point in turn indicates that nucleolin as well as other argyrophilic proteins can be very useful guides for biocompatible silver cluster preparation.

Contrary to much larger semiconductor quantum dots, which typically only enter cells by endocytosis and remain trapped in endosomes, live cells loaded with much smaller **P3-Ag** in Dulbecco's Modified Eagle's Medium (DMEM) at 37 °C and 5% CO_2 for 1 h show the silver nanocluster emission to be distributed evenly (Figure 3C, left). The **P3-Ag** also passes through the nuclear membrane, resulting in the weak nuclear staining. The methanol-fixed cells loaded with **P3-Ag** for 1 h (Figure 3C, right) show evenly distributed silver cluster emission in the cytoplasm and strong nuclear staining of some organelles. **P3-Ag** was also loaded into cells at 4 °C (Figure 3C, center), suggesting that the peptide-protected silver clusters do not enter by endocytosis, an important feature for further biological applications.

Received: October 17, 2006
Published online: February 7, 2007

Keywords: cell staining · fluorescence spectroscopy · imaging agents · peptides · silver

[1] K. J. Oparka, A. G. Roberts, S. Santa Cruz, P. Boevink, D. A. M. Prior, A. Smallcombe, *Nature* **1997**, *388*, 401.
[2] I. Chem, A. Y. Ting, *Curr. Opin. Biotechnol.* **2005**, *16*, 35.
[3] F. Stellacci, C. A. Bauer, T. Meyer-Friedrichsen, W. Wenseleers, S. R. Marder, J. W. Perry, *J. Am. Chem. Soc.* **2003**, *125*, 328.
[4] R. Breinbauer, M. Köhn, *ChemBioChem* **2003**, *4*, 1147.
[5] J. T. Petty, J. Zheng, N. V. Hud, R. M. Dickson, *J. Am. Chem. Soc.* **2004**, *126*, 5207.
[6] J. Zheng, R. M. Dickson, *J. Am. Chem. Soc.* **2002**, *124*, 13982.
[7] L. Peyser-Capadona, J. Zheng, J. I. Gonzalez, T. H. Lee, S. A. Patel, R. M. Dickson, *Phys. Rev. Lett.* **2005**, *94*, 058301.
[8] U. Scheer, R. Hock, *Curr. Opin. Cell Biol.* **1999**, *11*, 365.
[9] C. Goodpasture, S. E. Bloom, *Chromosoma* **1975**, *53*, 37.
[10] M. Horky, V. Kotala, M. Anton J. Wesierska-Gadek, *Ann. N. Y. Acad. Sci.* **2002**, *973*, 258.
[11] P. Roussel, D. Hernandez-Verdun, *Exp. Cell Res.* **1994**, *214*, 465.
[12] B. K. Kennedy, M. Gotta, D. A. Sinclair, K. Mills, D. S. McNabb, M. Murthy, S. M. Pak, T. Laroche, S. M. Gasser, L. Guarente, *Cell* **1997**, *89*, 381.
[13] J. Yu, D. Parker, R. Pal, R. A. Poole, M. J. Cann, *J. Am. Chem. Soc.* **2006**, *128*, 2294.
[14] K. Dowling, M. J. Dayel, M. J. Lever, P. M. W. French, J. D. Hares, A. K. L. Dymoke-Bradshaw, *Opt. Lett.* **1998**, *23*, 810.
[15] L. A. Peyser, A. E. Vison, A. P. Bartko, R. M. Dickson, *Science* **2001**, *291*, 103.
[16] H. M. Bourbon, B. Lapeyre, F. Amalric, *J. Mol. Biol.* **1988**, *200*, 627.
[17] A. Henglein, *Chem. Rev.* **1989**, *89*, 1861.
[18] R. A. Wapnir, *Protein Nutrition and Mineral Absorption*, CRC Press, Boca Raton, FL, **1991**, p. 147.

