Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Fluorescence Visualization of Newly Synthesized Proteins in Mammalian Cells

Kimberly E. Beatty, Julie C. Liu, Fang Xie, Daniela C. Dieterich, Erin M. Schuman, Qian Wang, & David A. Tirrell

Experimental Section

Cell Culture

Untransfected mouse embryonic fibroblasts (MEF18) and MEF transfected with Su9-GFP (MEF-mitoGFP), a mitochondrially-localized enhanced GFP, were a gift from D.C. Chan (California Institute of Technology, Pasadena, CA).[1] Human mammary epithelial cells (MCF-10A), human embryonic kidney cells (HEK 293T), and Chinese hamster ovary cells transfected with the human α5 integrin subunit fused to GFP (CHO-α5) were provided by A.R. Asthagiri (Caltech).[2] HeLa cells were a gift from C.D. Smolke (Caltech). Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex BioSciences (Walkersville, MD). African green monkey kidney cells (COS-7) were purchased from American Type Culture Collection (Manassas, VA). MEF18, MEF-mitoGFP, HEK 293T, HeLa, and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA), 50 U/mL penicillin, and 50 μg/mL streptomycin. CHO-α5 cells were maintained in a similar medium supplemented with 1% (v/v) non-essential amino acid solution (Sigma-Aldrich, St. Louis, MO) and 1 mM sodium pyruvate. Near-confluent cells were passaged with 0.05% trypsin in 0.52 mM EDTA. HUVEC were grown in Endothelial Growth Medium-2 (EGM-2, 2% serum, Cambrex BioSciences) and passaged non-enzymatically by treatment with 0.61 mM EDTA.

Preparation of Cells for Fluorescence Microscopy

Near-confluent cells in 100 mm Petri dishes were rinsed twice with 5 mL warm phosphate-buffered saline (PBS). Cells were detached with either EDTA (HUVEC) or trypsin in EDTA and treated with 2 mL of SFM [DMEM, with 1 mg/mL bovine serum albumin (BSA, fraction V, Sigma-Aldrich), without Met] containing 2.3 mg/mL soybean trypsin inhibitor (Sigma-Aldrich). The cells were pelleted via centrifugation (200g, 3 min), washed with 3 mL of SFM, and counted. Cells were added at a density of 0.25×10^6 – 1×10^6 cells per well to prepared slides (0.36×10^6 – 1.43×10^6 cells/cm^2).

Lab-Tek II Chamber Slides (8-well, Nalge Nunc International, Rochester, NY) were prepared by treatment with fibronectin solution (10 μg/mL) at 4 °C overnight. The wells were rinsed three times with PBS, blocked with a 2 mg/mL solution of heat-inactivated BSA for 30 min at room temperature, and rinsed with PBS.

All cells were incubated for 30 min in SFM to deplete intracellular Met stores. Cycloheximide (50 μM) or anisomycin (40 μM) was added to control cells during this time to inhibit protein synthesis. After incubation, either 1 mM Met or 1 mM Hpg was added to the medium. After 4 h, wells were rinsed once with PBS and the medium was replaced with SFM containing 1 mM Met for the chase.

After a 2 h chase, cells were rinsed three times with warm PBS, fixed with 3.7% paraformaldehyde solution for 10 min, and rinsed twice with PBS. Cells that were stained with anti-nucleolar antibodies were permeabilized with 0.1% Triton X100 for 3 min. The cells were treated with a blocking solution [10% (v/v) fetal calf serum (Cambrex BioSciences), 50 mg/mL sucrose, 20 mg/mL BSA] for at least 30 min at room temperature and rinsed twice with PBS. For nucleolar staining, cells were incubated with antibody clone 125-10 (Chemicon, Temecula, CA) at a dilution of 1:40 for 1 h at room temperature. After rinsing three times with PBS, cells were incubated with a secondary antibody solution containing 3% BSA and 12.5 μg/mL Cy2-conjugated affinity-purified goat anti-mouse secondary antibody (Chemicon) for 1 h at room temperature. Cells were then rinsed three times with PBS.

Cells were dye-labeled as previously described.[3] Chamber wells were filled to the top with >1.2 mL of PBS (pH 7.5) containing 200 μM $CuSO_4$, 400 μM TCEP, 200 μM triazole ligand, and 25 μM 3-azido-7-hydroxycoumarin.[4] The wells were sealed with polyolefin tape (Nalge Nunc), wrapped in foil, and inverted. Inversion was necessary to prevent debris from forming on the slide surface. Slides were allowed to react on a waver at room temperature overnight.

After reaction, cells were washed four times with PBS (1% Tween 20, 0.5 mM EDTA) and once with water. Slides were agitated for 1 min between washes. Chamber walls were removed from the slide. Mounting medium was added, and a cover slip was attached before visualization.

Preparation of Cells for Flow Cytometry

As described above, pulse-labeling was performed directly in the 35- or 60-mm tissue culture polystyrene dishes in which cells were grown. After the chase, cells were washed twice with PBS and detached using 0.05% trypsin in EDTA. Cells were centrifuged (200*g*, 3 min), lightly fixed in a 1% paraformaldehyde solution for 10 min at room temperature, washed with PBS, and treated with a blocking solution for at least 30 min at room temperature. Cells were incubated at 4 °C overnight in PBS supplemented with 200 μM $CuSO_4$, 400 μM TCEP, 200 μM triazole ligand, and 25 μM 3-azido-7-hydroxycoumarin. For optimization of $CuSO_4$ concentration, cells from 60-mm plates were split and supplemented with 50–500 μM $CuSO_4$.

Fluorescence Microscopy

Fixed cells were imaged on a confocal microscope (Zeiss LSM 510 Meta NLO, Thornwood, NY) at Caltech's Biological Imaging Center. Each set of images was obtained with identical conditions to capture either GFP or coumarin fluorescence. To visualize GFP fluorescence or Cy2-labeled nucleoli, cells were excited at 488 nm (Argon laser) and emission was passed through a bandpass filter (500–550 nm) before imaging.

Coumarin fluorescence was obtained by two-photon excitation at 800 nm (Ti:sapphire laser) with emission collected between 376–494 nm. Coumarin fluorescence varied among the cell lines and the settings were optimized for each cell type through comparison to labeling from Met or Hpg–cycloheximide control cells. The final coumarin image represents the average of two scans. For insets (Figure 1a), the sum of four scans was taken. The GFP and coumarin images were superimposed and false-colored for the overlay image (Figure 1a, right column). All images were acquired with a Plan-Apochromat 63x/1.4 oil objective (Zeiss) and analyzed with Zeiss LSM software.

Flow Cytometry

After overnight treatment with the coumarin dye, cells were washed once with PBS (1% Tween 20, 0.5 mM EDTA), resuspended in a total volume of 500 μL of PBS, and filtered through a 50 μm Nytex nylon mesh screen (Sefar, Depew, NY). Cells were analyzed on a BD Bioscience FACSAria flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA) at Caltech's Flow Cytometry Facility. Coumarin fluorescence was excited by a 407 nm violet laser and detected after passage through a 450/40 bandpass filter. GFP fluorescence was excited by a 488 nm blue laser and detected after passage through a 530/30 bandpass filter. Unlabeled MEF18, coumarin-labeled MEF18, and MEF-mitoGFP without coumarin were analyzed to ensure minimal cross-over fluorescence in each channel. Three samples were prepared for each experiment, and 10,000 events were collected for each sample. Forward- and side-scatter properties were used to exclude doublets, dead cells, and debris from analysis. FlowJo 5.7.1 software (Tree Star, Inc., Ashland, OR) was used for data analysis. The mean fluorescence of each population was averaged to give the reported mean fluorescence. The error bars represent one standard deviation.

Bibliography

[1] H. C. Chen, A. Chomyn, D. C. Chan, *J. Biol. Chem.* 2005, *280*, 26185.
[2] G. P. Richman, D. A. Tirrell, A. R. Asthagiri, *J. Control. Rel.* 2005, *101*, 3.
[3] K. E. Beatty, F. Xie, Q. Wang, D. A. Tirrell, *J. Am. Chem. Soc.* 2005, *127*, 14150.
[4] Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless, M. G. Finn, *J. Am. Chem. Soc.* 2003, *125*, 3192.