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Fluorinated colloidal gold immunolabels for imaging select proteins in parallel with lipids using high-resolution secondary ion mass spectrometry

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Abstract

The local abundance of specific lipid species near a membrane protein is hypothesized to influence the protein's activity. The ability to simultaneously image the distributions of specific protein and lipid species in the cell membrane would facilitate testing these hypotheses. Recent advances in imaging the distribution of cell membrane lipids with mass spectrometry have created the desire for membrane protein probes that can be simultaneously imaged with isotope labeled lipids. Such probes would enable conclusive tests of whether specific proteins co-localize with particular lipid species. Here, we describe the development of fluorine-functionalized colloidal gold immunolabels that facilitate the detection and imaging of specific proteins in parallel with lipids in the plasma membrane using high-resolution SIMS performed with a NanoSIMS. First, we developed a method to functionalize colloidal gold nanoparticles with a partially fluorinated mixed monolayer that permitted NanoSIMS detection and rendered the functionalized nanoparticles dispersible in aqueous buffer. Then, to allow for selective protein labeling, we attached the fluorinated colloidal gold nanoparticles to the nonbinding portion of antibodies. By combining these functionalized immunolabels with metabolic incorporation of stable isotopes, we demonstrate that influenza hemagglutinin and cellular lipids can be imaged in parallel using NanoSIMS. These labels enable a general approach to simultaneously imaging specific proteins and lipids with high sensitivity and lateral resolution, which may be used to evaluate predictions of protein co-localization with specific lipid species.

INTRODUCTION

To coordinate the numerous biological processes that occur at the cell's surface, the plasma membrane is compartmentalized into compositionally and functionally distinct domains.¹ Knowledge of component distribution in the plasma membrane facilitates identifying the mechanisms that underlie biological function. Given their key roles in biological processes,

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ASSOCIATED CONTENT

Supporting Information. Experimental protocols for the ELISA assay of immunolabel binding, peak fitting of XPS spectra for assessment of nanoparticle composition, and statistical analysis of NanoSIMS data. Images of colorimetric test for primary amines on lysine/MUDA/PFDT-nanoparticles, nitrogen 1s XPS spectrum of lysine/MUDA/PFDT-nanoparticles, UV-vis spectrum of antibody-functionalized nanoparticles, SEM and NanoSIMS images of aggregated immunolabels, composite ¹⁹F⁻, ¹⁹⁷Au⁻, and secondary electron image of cell, and composite ¹⁹F⁻, ¹³C-enrichment, and secondary electron images of cells. This material is available free of charge via the Internet at <http://pubs.acs.org>.

a number of labeling tools have been developed for detecting and imaging proteins. This includes genetically encoded fluorescent protein constructs and functionalized antibodies that can be detected with fluorescence or electron microscopy.^{2–5} Use of these labels has revealed certain membrane proteins cluster during cell signaling and virus budding, suggesting synergistic protein action may be a general mechanism that is common to membrane-mediated processes.^{6–8}

Though important, protein clustering is only one aspect of the plasma membrane organization that influences biological processes. The local abundances of cholesterol and certain lipid species, such as sphingolipids and phosphatidylethanolamine, near membrane proteins are also reported to affect protein function.^{6, 8–11} To determine whether specific proteins are located within membrane domains that are enriched with particular lipid species, the protein and lipid species of interest must be visualized in parallel. However, imaging the lipid distribution in the plasma membrane is a significant challenge. The genetically encoded fluorescent tags and functionalized affinity labels that enable routine visualization of specific proteins with fluorescence or electron microscopy cannot be used to detect cholesterol or the majority of lipid species. Fluorescent lipid analogs can be visualized in parallel with membrane proteins labeled with genetically encoded fluorescent tags, but only a fraction of the lipids of interest will bear fluorophores, and these fluorophores may alter the localization of the labeled lipid.^{12–14}

Secondary ion mass spectrometry (SIMS) is one of the few approaches for directly imaging the lipid organization in biological membranes without the use of fluorophores.^{15–27} During SIMS analysis, a primary ion beam desorbs neutral and ionized molecules and molecular fragments from the sample's surface. The ionized species, which are called secondary ions, are analyzed by a mass spectrometer, and a mass spectrum of the molecules in the beam's focal area is generated. By scanning the analysis beam across the sample and collecting the resulting secondary ions, the intensities of the component-specific secondary ions detected at each position can be used to construct a map of the sample's surface composition. The distributions of lipids within cell membranes have been chemically imaged using time-of-flight SIMS (TOF-SIMS).^{16–18} Because TOF-SIMS can detect intact and slightly fragmented molecular ions, this molecular imaging SIMS approach does not require labels to identify the parent molecule. However, TOF-SIMS imaging of distinct lipid microdomains in cell membranes has not yet been achieved, presumably due to limited sensitivity and spatial resolution.

A SIMS instrument capable of mapping the isotopic and elemental composition at the sample surface with a lateral resolution as good as 50 nm (Cameca NanoSIMS 50) has enabled detecting and imaging submicrometer-size domains of isotopically labeled lipids in membranes.^{21, 23, 28–30} Because this instrument detects monoatomic and diatomic secondary ions, each species of interest must contain a distinct stable isotope so that the secondary ions generated during NanoSIMS analysis can be linked to the parent molecule.²¹ The distinct stable isotopes that encode for component identity can be selectively incorporated into cholesterol and specific lipid species with established metabolic labeling techniques.^{31–33} However, because proteins share the same amino acid building blocks, metabolic labeling cannot be used to selectively tag specific proteins with the orthogonal distinct isotopes or non-native elements that would permit them to be imaged in parallel to lipids with a NanoSIMS.

Existing approaches for selective protein labeling also have their limitations. Genetic methods for unnatural amino acid substitution³⁴ must be repeated for every protein of interest. Immunolabels have broader applicability, and commercially available colloidal gold-functionalized immunolabels produce distinctive ¹⁹⁷Au[–] secondary ions that can be

used to detect specific proteins with a NanoSIMS. Nevertheless, colloidal gold-functionalized immunolabels are not suitable for sensitive NanoSIMS detection in parallel with lipids. Specifically, the high magnetic field strength necessary to collect $^{197}\text{Au}^-$ secondary ions with the magnetic sector mass spectrometer on the NanoSIMS 50 precludes simultaneous detection of closely spaced masses, such as major and minor stable isotopes like $^{12}\text{C}^{14}\text{N}^-$ and $^{12}\text{C}^{15}\text{N}^-$, $^{13}\text{C}^1\text{H}^-$ and $^{12}\text{C}^1\text{H}^-$, and even $^{12}\text{C}_2^-$ and $^{12}\text{C}^{14}\text{N}^-$. As a result, the ^{15}N - and ^{13}C -enrichment that is used to detect ^{15}N - and ^{13}C -labeled lipids, respectively cannot be imaged in parallel with the $^{197}\text{Au}^-$ ions. Therefore, to image $^{197}\text{Au}^-$ and the lipid-related stable isotopic ratios, the magnetic field would have to be switched so that ions with different mass-to-charge ratios (m/z) could be consecutively collected on a detector. This approach reduces the measurement sensitivity because the $^{197}\text{Au}^-$ and lipid-specific ions are continuously sputtered from the sample, but only a subset of each ion species is collected at any time. As a result, few counts of each species is collected for a given depth of analysis, which in the case of the lipid membrane, is relatively shallow (<7 nm).

Antibodies functionalized with a distinctive and low molecular weight isotope or element could permit imaging specific proteins in parallel to lipids with a NanoSIMS. Fluorine is an attractive candidate elemental label due to its low abundance in biological materials and high secondary ion yields.^{21, 28} Methods to conjugate small fluorine-containing molecules to antibodies have been developed for positron-emission tomography applications.^{35, 36} However, we experimentally found that functionalizing antibodies with the high numbers of fluorinated molecules that enable sensitive detection with NanoSIMS compromised antibody binding.

Here we report the development of a functionalized immunolabel for the detection of specific proteins in parallel with metabolically labeled lipids using NanoSIMS. The immunolabel consists of a colloidal gold nanoparticle that is functionalized with a partially fluorinated mixed monolayer and conjugated to a secondary antibody that permits selective protein labeling. The partially fluorinated mixed monolayer on the nanoparticle contains a large number of low mass, high yielding $^{19}\text{F}^-$ secondary ions, which permits highly sensitive NanoSIMS detection without compromising the simultaneous detection of lipid-specific secondary ions.^{21, 28} As a proof of concept, we have used this immunolabel to detect and image influenza hemagglutinin in parallel with isotope-labeled lipids in the plasma membranes of cells with a NanoSIMS. Hemagglutinin was selected as the protein of interest because its spatial organization in the membranes of transfected fibroblast cells is well-characterized.^{37, 38} In this report, we image all cellular lipid species due to the ease of isotopically labeling them, and they permit demonstrating that the fluorinated gold immunolabels can be detected in parallel to the minor and major stable isotopes that are used to visualize the lipid-specific isotope enrichment with a NanoSIMS.

MATERIALS AND METHODS

Materials

Uniformly carbon-13 labeled algal fatty acids (98 atom% carbon-13) were from Cambridge Isotope Laboratories. N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) was purchased from Prochem Inc. Hydrogen tetrachloroaurate(III) trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), Lipid-reduced fetal bovine serum (LR-FBS), and calf serum were from HyClone Laboratories. Poly-L-lysine and chemical fixation reagents were from Electron Microscopy Sciences. Fatty acid-free bovine serum albumin (BSA), goat anti-mouse polyclonal antibody, $^{13}\text{C}_{18}$ -stearic acid (99 atom% carbon-13), and all other reagents were purchased from Sigma-Aldrich. Primary antibody (FC 125 anti-hemagglutinin developed in mouse) and the cell line that expresses influenza hemagglutinin (Clone 15 cell line) was the generous gift from

Joshua Zimmerberg (Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD).

Synthesis of Gold Nanoparticles

2.9 mL (6.4 mmol) of oleylamine was dissolved in 49 mL of toluene and brought to a slow reflux. $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (24.3 mg, 0.062 mmol) was dissolved in 1.2 mL (3.7 mmol) of oleylamine and 1 mL of toluene. The gold solution was added to the refluxing toluene solution, refluxed for 2 h, and cooled to room temperature. Particles were purified by precipitation with 100 mL of methanol and centrifugation at 3000 rpm for 20 min. The particles were rinsed twice with methanol (50 mL) and dissolved in toluene (10 mL), producing a bright red colloidal solution.

Thiol Modification of Gold Nanoparticles

A solution of gold nanoparticles in toluene (3 mL) was added to 1H,1H,2H,2H-perfluorodecanethiol (8.5 μL , 0.029 mmol) in toluene (0.1 mL). After 2 min, 11-mercaptoundecanoic acid (44.8 mg, 0.205 mmol) in toluene (0.4 mL) was added, resulting in a concentration of 8.3 mM 1H,1H,2H,2H-perfluorodecane thiol and 58.6 mM 11-mercaptoundecanoic acid. The reaction was stirred at room temperature for 18 h. Excess thiols were removed by pelleting the particles by centrifugation, decanting the supernatant, and resuspending the particles in 1.5 mL toluene. After repeating this process three times, the particles were resuspended in 1 mL of DMSO and stored at 4 °C. The particles were stable for at least one month under these conditions and did not exhibit irreversible flocculation.

XPS of Nanoparticles

XPS was performed on a Kratos Axis ULTRA with a monochromatic aluminum source, run at 150 W (15 kV) and spherical capacitor electron energy analyzer. The Kratos has a take-off angle of 90 degrees with respect to the sample plane with pass energies of 160 eV for surveys and 40 eV for high resolution. Spectra were calibrated using the carbon 1s peak at 285.0 eV. See SI for details of fitting XPS spectra for assessment of nanoparticle composition.

Qualitative Assessment of Nanoparticle Dispersibility in Aqueous Solution

To evaluate the dispersal of the nanoparticles in aqueous media, the particles were pelleted by centrifugation, the toluene was decanted, and phosphate buffered saline (PBS) was added. After brief sonication, particle dispersal was visually assessed. Solutions of well-dispersed particles were red in color. Poor dispersibility of the particles was evidenced by the solution's color changing from red to purple or blue, and the presence of particle precipitation.^{39, 40}

Lysine Modification of Gold Nanoparticles

Sulfo-NHS (44.0 mg, 0.205 mmol) and N,N'-dicyclohexylcarbodiimide (DCC) (59.3 mg, 0.287 mmol) were dissolved in 3 mL of DMSO and 0.68 mL of ethanol. Thiol-modified particles in DMSO (1 mL) were added to the sulfo-NHS and DCC solution and stirred for 3 h. Particles were pelleted by centrifugation, and resuspended in 1 mL of ethanol. Ethanol (4 mL) was added to lysine (1.68 g, 10.3 mmol) in 1.5 mL of water. 1 mL of particles modified with sulfo-NHS in ethanol was added to the lysine solution and stirred overnight. Excess lysine was removed by pelleting the particles with centrifugation, decanting the supernatant, and resuspending the particles in deionized water. After repeating this process four times, the particles were stored at room temperature in water until protein conjugation. Particles stored under these conditions for one year did not exhibit irreversible flocculation.

TOF-SIMS of Functionalized Nanoparticles

A PHI Trift-III TOF-SIMS (Physical Electronics Incorporated, Chanhassen, MN) instrument with a $^{197}\text{Au}^+$ liquid ion gun operated at 22 kV was used to collect positive-ion spectra in unbunched mode. The primary ion dose for each analysis was 3×10^{13} ions/cm². Twelve spectra were acquired from each set of thiol-functionalized nanoparticles. One of the twelve spectra of the nanoparticles modified with PFDT and MUDA (MUDA/PFDT-nanoparticles) was excluded from further analysis due to very low signal intensity. Each peak in a spectrum was normalized to the total intensity of all the peaks. Representative spectra were created by averaging the normalized intensities of the peaks in the 11 spectra of the MUDA/PFDT-nanoparticles and the 12 spectra of the nanoparticles that had lysine conjugated to the MUDA and PFDT monolayer (lysine/MUDA/PFDT-nanoparticles).

Ovalbumin and Antibody Oxidation and Reaction with Nanoparticles

Ovalbumin (5 mg) was dissolved in 5 mL of 0.1 M Na_2HPO_4 buffer at pH 7.5. 20 μL of 0.1 M NaIO_4 was added to 200 μL of ovalbumin solution, inverted several times, and the oxidation was allowed to proceed for 30 min in the dark. 60 μL of goat anti-mouse antibody solution (2.2 mg/mL) was desalted into 120 μL of 0.1 M Na_2HPO_4 buffer at pH 7.5 using Amicon Ultra-4 10K centrifuge filters, producing desalted antibody at a concentration of 1.1 mg/mL. 12 μL of 0.1 M NaIO_4 was added to the desalted antibody (120 μL), the reaction vial was inverted several times, and the oxidation was allowed to proceed for 30 min in the dark. A solution of oxidized goat anti-mouse polyclonal antibody (92.5 μL , 1.0 mg/mL) was added to 250 μL of lysine/MUDA/PFDT-nanoparticles in water and gently shaken. After 5 min, 111 μL of oxidized ovalbumin solution was added to the particles, gently shaken, and incubated overnight. To remove unconjugated protein, the functionalized particles were diluted in 700 μL of 0.1 M Na_2HPO_4 buffer (pH 7.5) containing 2% BSA, the solution was centrifuged at 5000 rpm for 20 min, the supernatant was removed, and fresh 2% BSA in buffer was added. This process was repeated a total of six times.

Evaluation of Aggregation of Antibody-Functionalized Particles with UV-Vis

Absorbance measurements were performed on an Agilent 8453 UV-Visible Spectrophotometer. Antibody-functionalized particles were diluted 1:1 with 0.1 M Na_2HPO_4 buffer (pH 7.5), and filtered through a 0.1 μm syringe filter prior to measurement. Additional buffer was added to the functionalized gold immunolabel solution to obtain surface plasmon resonance absorbance below unity.

ELISA Assay of Immunolabel Binding

Antibody binding affinity was assessed by ELISA using established protocols.⁴¹ See also SI.

Cell Culture

Cells were grown in standard cell growth medium consisting of high glucose Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% calf serum, 10^4 U/mL penicillin G, and 10 mg/mL streptomycin. Silicon substrates were placed in a cell culture dish when the cells were passaged and incubated overnight to permit cell attachment to the substrates. To metabolically label the cellular lipids with carbon-13, Clone 15 cells were passaged into DMEM supplemented with 1% calf serum, 10% LR-FBS, 215 μM ^{13}C -fatty acids (4:1:28 mass ratio of UL- ^{13}C - algal fatty acids/ ^{13}C -stearic acid/fatty acid-free BSA), 50 μM cholesterol, and 20 μM ethanolamine. The growth medium was supplemented with ^{13}C -fatty acid mixture and ethanolamine each day. On day 3, the cells were passaged. Cells (2×10^6 cells) were cultured in dishes containing fresh medium, supplements, and sterilized and poly-L-lysine-coated 5 x 5 mm silicon substrates for one day.

Analysis of ^{13}C -Fatty Acid Incorporation

Cells attached to the culture dish were used to assess the incorporation of ^{13}C -fatty acids into the cellular lipids. Cells were washed with PBS, detached from the dish, and collected via centrifugation. The lipids were extracted as reported,⁴² and saponified with ethanolic KOH at 70 °C for 45 min. After acidification with HCl, the fatty acids were extracted into hexane and dried under N_2 (g). The residue was dissolved in $\text{MeOH}:\text{CH}_2\text{Cl}_2$ (1:2), and reacted with 2 M trimethylsilyldiazomethane for 60 min. The composition of the resulting fatty acid methyl esters (FAME) was assessed with a GC/MS system consisting of an Agilent 7890 gas chromatograph, an Agilent 5975 mass selective detector and HP 7683B (Agilent Inc, Palo Alto, CA) autosampler. Gas chromatography was performed on a ZB-WAX (30 m $\times$ 0.25 mm I.D., 0.25 μm film thickness) capillary column (Phenomenex, Torrance, CA). Samples (1 μL) were injected in split mode (5:1) to the GC/MS system. The mass spectrometer was operated in positive electron impact mode at 69.9 eV in the m/z 50–800 scan range, which was used to detect six FAMES: C16:0, C16:1, C18:0, C18:1, and C18:2. Spectra were evaluated using the HP Chemstation (Agilent) program. Identification was performed using mass spectra obtained from authentic standards and additionally confirmed with NIST08 and W8N08 libraries (John Wiley & Sons, Inc., USA).

Immunolabeling and Preservation

Substrates with adherent cells were rinsed twice each with PBS with calcium and magnesium and Hendry's phosphate buffer (HPB), and fixed for 10 min with 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M HPB. Cells were rinsed twice with HPB, once with PBS without calcium and magnesium (PBS -M), blocked with immunowash buffer (PBS -M with 1% BSA), and incubated with 15 μL of 0.18 mg/mL primary antibody for 30 min. Excess primary antibody was removed with by rinsing with immunowash buffer. The functionalized colloidal gold immunolabels were diluted 1:1 with 0.1 M Na_2HPO_4 buffer (pH 7.5) and filtered with a 0.1 μm syringe filter. Samples were incubated with 25 μL functionalized immunolabels for 30 min and rinsed twice with immunowash buffer and HPB. Cells were fixed with 4% glutaraldehyde in HPB for 30 min, washed twice with HPB and once with water, post-fixed with 1% OsO_4 for 15 min, rinsed three times with water, and air dried. Negative controls were prepared using immunowash buffer in place of the primary antibody.

SEM

Sample quality was assessed by imaging the cells at low magnification (500 – 1100 $\times$) using a Hitachi 4800 SEM with 1 kV accelerating voltage. The immunolabels on the cells were imaged on a JEOL 7000F with 15 kV accelerating voltage using a backscatter detector.

NanoSIMS Analysis

To prevent sample charging during SIMS analysis, the samples were coated with ~3 nm of iridium. While the depth of analysis employed in some of the following experiments did not exceed the thickness that the metal coating would be on an atomically flat surface, we empirically observed that the lipid-specific secondary ions in these experiments are detected at a more shallow sputtered depth than would be expected. We interpret this result to indicate that sample surface irregularity and metal/membrane mixing on a length scale below the lateral resolution of these SIMS measurements (< 100 nm) leaves the lipid more exposed than the apparent metal coating would indicate. High resolution SIMS was performed on a NanoSIMS 50 (Cameca, France) at Lawrence Livermore National Laboratory (Livermore, CA). Measurements were made with a 16 keV $^{133}\text{Cs}^+$ primary ion beam. Confirmation that the fluorine signal located the functionalized immunolabels was performed using a ~1.37 pA primary ion beam and simultaneously collecting the $^{19}\text{F}^-$

and $^{197}\text{Au}^-$ secondary ions and secondary electrons. Five replicate scans of 256 by 256 pixels with a dwell time of 1 ms/pixel were acquired for each 20 by 20 μm^2 analysis region, resulting in a pixel size of 78 by 78 nm^2 and a primary ion dose of 7.2×10^{14} ions/ cm^2 . The analysis depth was approximately 3 nm, calculated as reported using the sputtering rate of $2.5 \text{ nm} \cdot \mu\text{m}^2/\text{pA} \cdot \text{s}$ that was determined on other biological samples.⁴³ For the simultaneous imaging of the hemagglutinin protein and cellular lipids, the $^{12}\text{C}^1\text{H}^-$, $^{13}\text{C}^1\text{H}^-$, and $^{19}\text{F}^-$ secondary ions and secondary electrons were simultaneously collected using a ~ 0.264 pA primary ion beam. A mass resolving power of $\sim 6,700$ was used to separate isobaric interferences from the isotopes of interest. Four replicate scans of 256 by 256 pixels with a dwell time of 1 ms/pixel were acquired for each 15 by 15 μm^2 analysis region, resulting in a pixel size of 59 by 59 nm^2 and a primary ion dose of 1.9×10^{14} ions/ cm^2 . Each analysis overlapped with the preceding analysis region in order to facilitate stitching the resulting NanoSIMS images together in a mosaic. Images were cropped to show the region that did not overlap with a preceding analysis region. We calculated an analysis depth of approximately 0.8 nm using the sputtering rate of $2.5 \text{ nm} \cdot \mu\text{m}^2/\text{pA} \cdot \text{s}$ for biological samples.⁴³

NanoSIMS Image Analysis

A custom software package (L'IMAGE, L.R. Nittler, Carnegie Institution of Washington) run on the PV-Wave platform (Visual Numerics) was used to process the NanoSIMS data. $^{19}\text{F}^-$ images and ^{13}C -enrichment images that show the distributions of the fluorothiolate-functionalized colloidal gold immunolabels that locate the influenza hemagglutinin and the ^{13}C - lipids, respectively, were constructed using a 3 x 3-pixel boxcar smoothing algorithm, producing images with 176 nm lateral resolution. The ^{13}C -enrichment images were constructed by ratioing the $^{13}\text{C}^1\text{H}^-$ to the $^{12}\text{C}^1\text{H}^-$ secondary ion counts collected at every pixel, and then dividing that ratio by the natural abundance ratio (0.011237). Ratioing the $^{13}\text{C}^1\text{H}^-$ counts to the $^{12}\text{C}^1\text{H}^-$ counts minimizes signal variations induced by sample topography, producing a quantitative measure of the local enrichment in ^{13}C -labeled molecules compared to an unlabeled (natural abundance) cell.⁴⁴ The resulting ^{13}C -enrichment was color coded and mapped onto its corresponding pixel in the NanoSIMS image. See the SI for details of the statistical analysis of the NanoSIMS data.

RESULTS

Synthesis of the fluorothiolate-functionalized colloidal gold immunolabel

Our strategy for immunolabel synthesis involved functionalizing the colloidal gold nanoparticle with a partially fluorinated mixed monolayer, and then conjugating the fluorinated nanoparticle to secondary antibodies (Figure 1). We selected a bioconjugation strategy in which aldehydes are generated on the antibody's glycosylated, non-binding, Fc region for covalent reaction with amines on the nanoparticle, yielding a stable linkage that does not obstruct antibody binding.⁴⁰ To permit antibody attachment and to help render the particles dispersible in aqueous buffers, the thiols in the partially fluorinated mixed monolayer on the colloidal gold nanoparticle should have a chain length longer than seven methylene units and be capped with an ionizable amine moiety.^{39, 45, 46} Because amine-terminated perfluoroalkanethiols of sufficient length were not commercially available, we created a mixed monolayer that consisted of a fluorinated alkane thiol (1H,1H,2H,2H-perfluorodecanethiol, PFDT) for NanoSIMS detection, and a carboxylic-acid-terminated thiol (11-mercaptoundecanoic acid, MUDA) that could be converted to an ionizable terminal amine by reaction with lysine.⁴⁷ We anticipated that the hydrophobic PFDT component might induce the resulting functionalized nanoparticles to flocculate in water, as might be nanoparticles modified with a PFDT monolayer are not dispersible in aqueous solvent mixtures.⁴⁸ However, the presence of an ionizable terminal moiety on the capping monolayer promotes nanoparticle dispersibility in water,^{39, 45, 46} so we hypothesized that the

ratio of MUDA to PFDT in the monolayer could be adjusted to produce water-dispersible and partially fluorinated nanoparticles.

Gold nanoparticles with an average diameter of 20 nm were synthesized using oleylamine to reduce Au^{3+} in toluene.⁴⁹ This nanoparticle size is a compromise between large particles that facilitate secondary characterization of cell labeling with SEM, and smaller particles that typically have higher immunolabeling efficiency.⁵⁰ To identify the reaction conditions that produced water-dispersible and fluorinated nanoparticles, we varied the molar ratio of MUDA to PFDT between 1:10 and 10:1, and assessed the particles' fluorine content and dispersibility in aqueous buffer. XPS measurements indicated that MUDA to PFDT ratios of 1:10 and 1:1 yielded fluorinated colloidal gold nanoparticles, but fluorine was not detected on the nanoparticles modified with a 10:1 ratio of MUDA to PFDT (Table 1). Nanoparticle dispersibility in aqueous buffer followed the opposite trend; only the nanoparticles modified with a 10:1 ratio of MUDA to PFDT exhibited adequate dispersion in PBS, signified by the solution's red color. The poor dispersibility of particles modified with 1:1 and 1:10 ratios of MUDA to PFDT was evident from the solution's color changing from red to purple or blue, and visible particle precipitation.^{39, 40} Subsequent investigations of MUDA to PFDT ratios between 1:1 and 10:1 indicated that a 7:1 ratio reproducibly produced water-dispersible and partially fluorinated nanoparticles when PFDT was allowed to react with the colloidal gold for 2 minutes prior to MUDA addition (Table 1). Finally, amine functionalities were generated on the surface of the nanoparticles that had been functionalized with a partially fluorinated mixed monolayer (MUDA/PFDT-nanoparticles) by condensing the terminal carboxylic acids on the monolayer with lysine.

The presence of lysine on the surface of the MUDA/PFDT-nanoparticles that had been reacted with lysine (lysine/MUDA/PFDT-nanoparticles) was verified with three methods. First, a fluoresceinamine-based colorimetric test confirmed the presence of primary amines that are characteristic of lysine on the lysine/MUDA/PFDT-nanoparticles, but not on the unmodified MUDA/PFDT-nanoparticles (Figure S1). Second, comparison of the XPS spectra of lysine/MUDA/PFDT-nanoparticles and MUDA/PFDT-nanoparticles demonstrated the presence of chemical bonds distinctive of lysine conjugation to the nanoparticles (Figure 2). Specifically, the nitrogen 1s peaks at 399.4 eV (C-NH_2) and 401.3 eV (N-C=O) that appeared in the XPS spectra of lysine/MUDA/PFDT-nanoparticles (Figure 2A and Figure S2) are indicative of the amines on the lysine residue and the formation of an amide bond with lysine, respectively. Additionally, the increase and shift in the carbonyl peak's maxima from 288.8 eV in the MUDA/PFDT-nanoparticle spectra to 288.3 eV in the lysine/MUDA/PFDT-nanoparticle spectra signified the presence of the carboxylic acid on lysine and amide bond formation (Figure 2B and 2C). Furthermore, the shoulder at 286.4 eV that appeared in the lysine/MUDA/PFDT-nanoparticle spectra is characteristic of the C-N single bonds in lysine (Figure 2C). Based on deconvolution of the carbonyl peak shown in Figure 2C into separate carboxylic acid and amide peaks, more than 90% of the MUDA carboxylic acids were covalently attached to lysine. Third, comparison of TOF-SIMS spectra of lysine/MUDA/PFDT-nanoparticles and MUDA/PFDT-nanoparticles demonstrated that lysine was covalently attached to the lysine/MUDA/PFDT-nanoparticles. Representative positive ion spectra of lysine/MUDA/PFDT-nanoparticles and MUDA/PFDT-nanoparticles are shown in Figure 3, where a range from m/z 1 to 300 is shown because ions with $m/z > 300$ have low detection efficiency with our TOF-SIMS instrumentation. Peaks that most likely correspond to gold (m/z 197), fluorinated PFDT fragment ions (m/z 69, 268, and 300 from CF_3^+ , $\text{C}_5\text{F}_{11}^+$, and $\text{C}_6\text{F}_{12}^+$, respectively), and MUDA fragment ions (m/z 41, 55, 243, and 296 from C_3H_5^+ , C_4H_7^+ , CH_2SAu^+ , and $\text{C}_5\text{H}_7\text{SAu}^+$, respectively) were visible in the spectra of the MUDA/PFDT-nanoparticles (Figure 3A). Additional peaks that likely correspond to lysine fragment ions (m/z 56, and 84 from $\text{C}_3\text{H}_6\text{N}^+$ and $\text{C}_5\text{H}_9\text{N}^+$, respectively)⁵¹ and the sodium adduct of a lysine-amide

fragment ion (m/z 224 from $C_9H_{17}N_2O_3Na^+$) were present in the spectra of the lysine/MUDA/PFDT-nanoparticles (Figure 3B). Although peak m/z 147 is often attributed to lysine,⁵¹ its co-appearance with peak m/z 73 in the lysine/MUDA/PFDT-nanoparticle spectra may instead indicate the presence of polydimethylsiloxane, a common surface contaminant.⁵² Peaks with m/z between 200 and 300 that correspond to MUDA and PFDT fragment ions are not present in the lysine/MUDA/PFDT-nanoparticle spectra, possibly due to the suppression of these ions by the more basic lysine residues.⁵³ Altogether, these experiments confirm that lysine had been covalently attached to the MUDA/PFDT-nanoparticles.

Immunolabel synthesis was completed by attaching the fluorinated nanoparticles to secondary antibodies. We desired to limit the amount of antibody conjugated to each fluorinated colloidal gold nanoparticle in order to prevent cross-linking the antigens on the cell surface, but low protein coverage induces nanoparticle aggregation.⁴⁰ To obtain the high protein surface coverage that stabilizes the nanoparticles in buffered solution without increasing the antibody loading, we first reacted the nanoparticles with a small amount of oxidized antibody, and then blocked the nanoparticle surface with ovalbumin, a small, glycosylated protein. This was accomplished by mildly oxidizing the polyclonal goat anti-mouse antibodies and ovalbumin with sodium periodate, generating reactive aldehyde moieties on the glycosylated regions of the proteins.⁴⁰ These aldehydes were then reacted with the amines on the functionalized nanoparticles under non-denaturing conditions. The resulting fluorothiolate-functionalized colloidal gold immunolabels exhibited a UV-vis absorption maximum at ~525 nm (Figure S3), indicating the absence of nanoparticle aggregation.⁴⁰ The affinity and selectivity of the immunolabels to their antigen, mouse antibody, was confirmed with ELISA.

Verification of cell labeling with fluorothiolate-functionalized colloidal gold immunolabels

We evaluated the detection of the fluorothiolate-functionalized colloidal gold immunolabels that located specific proteins on cells with scanning electron microscopy (SEM) and high-resolution SIMS performed with a NanoSIMS 50. As stated in the Introduction, we focused on the detection of influenza hemagglutinin in the plasma membranes of transfected fibroblast cells (Clone 15 cell line) in these experiments because the spatial organization of this protein is well-characterized.^{37, 38} Influenza hemagglutinin in the plasma membranes of Clone 15 cells was selectively labeled with a mouse anti-hemagglutinin monoclonal primary antibody, followed by the fluorothiolate-functionalized colloidal gold immunolabels. The cells were chemically fixed and imaged with SEM (Figure 4) and NanoSIMS (Figure 5). As a negative control experiment, we also used SEM to image a Clone 15 cell that was not labeled with primary antibody prior to exposure to the fluorothiolate-functionalized colloidal gold immunolabels (negative control).

SEM imaging was performed using backscattered electron (BSE) mode to permit unambiguous detection of the colloidal gold nanoparticles, as elements with high atomic weight, such as gold, appear bright in BSE images. The fluorothiolate-functionalized colloidal gold immunolabels that located the influenza hemagglutinin were clearly visible on the surface of the immunolabeled cell (Figure 4A). Though individual gold nanoparticles were present, the majority of the immunolabels were clustered in two dimensions on the cell surface. This two-dimensional clustering of the colloidal gold immunolabels that locate hemagglutinin has been previously reported by others, and it is indicative of hemagglutinin accumulation within small domains in the plasma membrane.^{37, 38} In contrast, large immunolabel aggregates, such as those that occur when ovalbumin is not used to block the surfaces of the nanoparticles (Figure S4), are significantly larger and have a three-dimensional appearance. Thus, the two-dimensional clusters of fluorothiolate-functionalized

colloidal gold immunolabels that we observed on the immunolabeled cell were indicative of hemagglutinin's native clustered distribution in the cell membrane, and not immunolabel aggregation. Noteworthy, very few individual particles (Figure 4B, closed arrows) or immunolabel clusters (Figure 4B, open arrows) were visible on the negative control Clone 15 cell that was not labeled with primary antibody prior to treatment with the functionalized immunolabels. Thus, very little nonspecific binding occurred.

Four regions on an immunolabeled Clone 15 fibroblast cell were analyzed with NanoSIMS to confirm that the fluorine secondary ion signal could be used to detect the fluorothiolate-functionalized colloidal gold immunolabels. NanoSIMS analysis was performed on cells that had not been imaged with SEM in backscattered electron mode because our earlier experiments indicated that electron impact at >5 kV during backscatter imaging decomposed the fluorinated alkane thiols on the nanoparticles. As shown below, SEM imaging performed at low magnification with low voltage (< 5 kV), however, permitted both evaluation of cell preservation²⁶ and NanoSIMS detection of the fluorinated alkane thiols. A representative NanoSIMS secondary electron image that was acquired in parallel to the secondary ion images shows the morphology of the immunolabeled cell (Figure 5A). Individual nanoparticles are not visible in the secondary electron image because the nanoparticles were smaller than the lateral resolution of this NanoSIMS analysis (approximately 100 nm). The inability to detect nanoparticle aggregates in this image indicates that large (> 100 nm) aggregates of the functionalized immunolabels were not present. However, local elevations in the immunolabel-specific $^{197}\text{Au}^-$ and $^{19}\text{F}^-$ secondary ions are clearly visible in the NanoSIMS secondary ion images (Figures 5B and 5C, respectively). The local elevations in the $^{197}\text{Au}^-$ and $^{19}\text{F}^-$ secondary ions varied in intensity and often had multi-pixel dimensions, which suggests they represent the two-dimensional clusters of differing numbers of individual immunolabels that we observed with SEM. Statistical analysis of the ion signal intensities detected at 60,180 1-by-1-pixel subregions within the representative region that corresponded to the surface of the cell indicated that $^{19}\text{F}^-$ and $^{197}\text{Au}^-$ ion counts/pixel greater than 32 and 18, respectively, were statistically significant local elevations. As implied by the overlay of the $^{197}\text{Au}^-$ and $^{19}\text{F}^-$ ion images (Figure S5), this statistical analysis also confirmed that the $^{197}\text{Au}^-$ and $^{19}\text{F}^-$ ion signals were positively correlated (correlation coefficient = 0.87 at 95% confidence interval). The $^{197}\text{Au}^-$ and $^{19}\text{F}^-$ ion signals detected on the cell surface at the three other analysis regions were also positively correlated (correlation coefficients = 0.83, 0.67, and 0.86 for 42,262, 56,911, and 50,138 1-by-1-pixel regions, respectively, at 95% confidence interval), confirming that the $^{19}\text{F}^-$ signal was produced by the fluorothiolate-functionalized colloidal gold immunolabels. Altogether, these results demonstrate that the functionalized immunolabels could be detected with NanoSIMS via local elevations in the $^{19}\text{F}^-$ or $^{197}\text{Au}^-$ secondary ions.

Simultaneous imaging of influenza hemagglutinin and all cellular lipid species in the cell membrane

Next, we assessed the ability to image the fluorothiolate-functionalized colloidal gold immunolabels that locate hemagglutinin in the plasma membranes of Clone 15 cells in parallel with lipids using a NanoSIMS. Again, we chose to image all cellular lipid species due to the ease of incorporating commercially available uniformly ^{13}C -labeled fatty acids into cellular lipids, and the resulting ^{13}C -lipids permitted demonstrating that the $^{13}\text{C}^1\text{H}^-$ and $^{12}\text{C}^1\text{H}^-$ ions used to measure the lipid-specific ^{13}C -enrichment could be simultaneously detected with the fluorinated immunolabels. The Clone 15 cells we studied had been metabolically labeled such that approximately 55% and 90% of the fatty acids in the cellular glycolipids and sphingomyelin, respectively, were uniformly carbon-13 labeled prior to tagging the influenza hemagglutinin with the fluorinated immunolabels. As a negative control, we also analyzed metabolically labeled Clone 15 cells that were not exposed to

primary anti-hemagglutinin antibody prior to the fluorothiolate-functionalized colloidal gold immunolabels. We imaged the fluorothiolate-functionalized colloidal gold immunolabels and ^{13}C -lipids in parallel by using NanoSIMS to simultaneously collect the $^{19}\text{F}^-$, $^{13}\text{C}^1\text{H}^-$ and $^{12}\text{C}^1\text{H}^-$ secondary ions. To minimize the fraction of secondary ions collected from the cytoplasm, NanoSIMS was performed with a lower primary ion dose than that used to evaluate immunolabel detection, resulting in a reduced secondary ion signal intensity compared to those obtained in Figure 5.

The secondary electron, $^{19}\text{F}^-$ ion, and ^{13}C -enrichment NanoSIMS images shown in Figure 6 are representative examples of the >50 other regions that we analyzed on five different cells (prepared on 2 separate days). Neither individual nanoparticles nor nanoparticle aggregates are visible in the NanoSIMS secondary electron images, confirming that large (> 100 nm) immunolabel aggregates were not present on the cells (Figure 6A and 6D). The locations of the hemagglutinin in the membrane are revealed by the regional elevations in the $^{19}\text{F}^-$ secondary ion counts that are visible in the representative NanoSIMS $^{19}\text{F}^-$ ion images of the immunolabeled cell (Figure 6B). Analysis of 79,596 1-by-1-pixel ROIs that corresponded to regions on the surface of the immunolabeled cell that did not overlap with a preceding NanoSIMS measurement indicated that $^{19}\text{F}^-$ counts/pixel greater than 3 were statistically significant local elevations. In comparison, very little non-specific labeling occurred on the negative control cell, as evidenced by the low number of pixels in which the $^{19}\text{F}^-$ secondary ion count exceeded unity (Figure 6E). The distribution of ^{13}C -lipids in the membrane of the immunolabeled and negative control cell are shown by the quantitative ^{13}C -enrichment images (Figure 6C and 6F) that were acquired in parallel to the $^{19}\text{F}^-$ ion images. The lateral variations in the ^{13}C -enrichment within the membrane suggest that the ^{13}C -lipids are unevenly distributed on the surface of both cells. Projection of the individual ion images onto the secondary electron image indicates that neither the immunolabels nor the ^{13}C -lipid distributions were correlated with cell topography (Figure S6A, S6B, S6D, and S6E). Similarly, the overlay of the $^{19}\text{F}^-$ ion and ^{13}C -enrichment NanoSIMS images (Figure S6C and S6F) indicates the immunolabels on the experimental cell were not co-localized with lipid-enriched or lipid-depleted regions of the membrane. The lack of a correlation between the $^{19}\text{F}^-$ counts and the ^{13}C -enrichment on the experimental cell was confirmed by statistical analysis (correlation coefficient = -0.03 at the 95% confidence interval). The absence of a significant negative correlation between the immunolabel- and lipid-specific secondary ion signals indicates the immunolabels did not hinder the detection of the underlying membrane. Overall, these imaging data demonstrate the ability to directly visualize the distribution of a specific protein, influenza hemagglutinin, with respect to membrane lipids and cell surface morphology.

DISCUSSION AND CONCLUSION

In a previous study, we used NanoSIMS to image the lipid distribution within phase-separated model membranes with high lateral resolution.²³ Here, we extended NanoSIMS analysis to simultaneously imaging all cellular lipid species and influenza hemagglutinin within the plasma membranes of intact cells by developing new fluorine-functionalized antibodies that permit selective protein detection in parallel with lipid-specific isotopes. This demonstration provides a general framework for simultaneously imaging distinct proteins and lipid species within the plasma membranes of cells with high sensitivity and lateral resolution. Because secondary antibodies are used to create the immunolabels, any protein of interest can be detected and imaged simply by employing the corresponding primary antibody. Though we imaged all of the cellular lipid species here as a proof of concept, the distribution of a protein of interest with respect to more specific lipid species may be achieved by employing established methods to incorporate distinct stable isotopes into phosphatidylethanolamine,⁵⁴ cholesterol,³¹ sphingolipids,³² or gangliosides.³³ This

approach may reveal whether the protein of interest is located within a membrane domain that is enriched with a particular lipid species when it is combined with cell preparation methods that do not induce reorganization of the lipid species.²⁶

Our immunolabel synthesis strategy was optimized to permit sensitive NanoSIMS detection and broad applicability. A key feature of the immunolabel design is that the fluorinated moieties were not directly attached to the antibody. Conjugation of the antibodies to gold nanoparticles that were capped with a partially fluorinated mixed monolayer permitted functionalizing the antibody with a high number of fluorine atoms without loss of binding. The strategy for immunolabel synthesis can be easily modulated to further broaden immunolabel applicability. For example, immunolabels that permit additional proteins to be detected in parallel to those labeled with fluorinated colloidal gold immunolabels can be fabricated by functionalizing the colloidal gold with alkane thiols that contain other non-native elements (i.e., bromine) or stable isotopes (i.e., deuterium or nitrogen-15). Immunolabels with smaller gold nanoparticles may be desirable for reduction of the steric hindrance between probes that may occur during the labeling procedure.⁵⁵ Such immunolabels may be created by employing the Brust method for nanoparticle synthesis.⁵⁶ We expect the label-specific secondary ions produced by smaller immunolabels will also be detectable by NanoSIMS even though these gold nanoparticles will be too small to be resolved in the NanoSIMS secondary electron images.

Use of the fluorothiolate-functionalized colloidal gold immunolabels described herein with metabolic incorporation of distinct stable isotopes and NanoSIMS analysis will facilitate the testing of predictions that specific membrane proteins are located within distinct lipid environments. By combining this approach to imaging membrane components with complementary assays of protein function, the role of the local lipid composition on protein function might also be elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Synthesis of functionalized immunolabel consisting of an antibody attached to a fluorinated gold nanoparticle (20 nm-diameter) functionalized with a partially fluorinated mixed monolayer. The gold nanoparticle is capped with a mixed monolayer composed of fluorinated and carboxylic acid-terminated thiols. The carboxylic acids are condensed with lysine, producing ionizable terminal amines. Aldehydes are generated on the glycosylated F_C region of the antibody and the glycosylated portions of the blocking protein, ovalbumin (not shown), by mild oxidation with sodium periodate. Reaction of the aldehydes on the antibody and ovalbumin with the amines on the fluorinated nanoparticles produces a stable linkage that does not interfere with antibody binding.

Figure 2.

XPS spectra of lysine/MUDA/PFDT-nanoparticles and MUDA/PFDT-nanoparticles. **(A)** Survey scan lysine/MUDA/PFDT-nanoparticles (red) and MUDA/PFDT-nanoparticles (blue) shows that lysine modification induced the appearance of the nitrogen 1s peaks at ~400 eV due to the amine and amide bonds that are characteristic of lysine coupling. The high-resolution spectrum of the nitrogen 1s peak from lysine/MUDA/PFDT-nanoparticles is shown in Figure S2. **(B)** The photoelectron spectra (blue) of the carbon 1s scan acquired from the MUDA/PFDT-nanoparticles was fit with the following carbon peaks: CF₂ (291.5 eV), COOH (288.8 eV), C-S (286.1 eV), and CH₂ (285.0 eV). **(C)** The photoelectron spectra (red) of the carbon 1s scan acquired from the lysine/MUDA/PFDT-nanoparticles was fit with the following carbon peaks: CF₂ (291.5 eV), COOH (288.5 eV), CONH (288.1 eV), C-N (286.5 eV), C-S (286.1 eV), and CH₂ (285.0 eV).

Figure 3. Representative positive ion TOF-SIMS spectra of (A) MUDA/PFDT-nanoparticles and (B) lysine/MUDA/PFDT-nanoparticles.

Figure 4.

SEM images of immunolabeled cells taken in backscatter mode. Elements with high atomic weight, such as gold, appear bright in backscatter mode images, which allows unambiguous identification of the functionalized colloidal gold immunolabels. **(A)** The fluorinated colloidal gold immunolabels visible on the cell surface locate the influenza hemagglutinin protein. The immunolabels were often arranged in two-dimensional clusters on the cell surface that are similar to the previously reported clusters of colloidal gold immunolabels that locate hemagglutinin, and are indicative of hemagglutinin clustering within the plasma membrane.^{37, 38} **(B)** A larger region of the surface of the negative control cell was imaged to acquire an overview of the extent of non-specific labeling. Few individual fluorinated gold immunolabels (closed arrows) and two very small immunolabel clusters (open-arrow) were detected on the Clone 15 cell that was not tagged with primary antibody prior to exposure to the fluorothiolate-functionalized colloidal gold immunolabels (negative control). The small amount of label present on the negative control sample indicated little non-specific binding occurred.

Figure 5.

NanoSIMS images were acquired of a 15 by 15 μm^2 region on a Clone 15 cell that was labeled with fluorothiolate-functionalized colloidal gold immunolabels that bound to the primary antibodies against the influenza hemagglutinin within the plasma membrane. **(A)** The secondary electron (SE) image shows cell morphology. The fluorothiolate-functionalized colloidal gold immunolabels are not visible in the secondary electron image because they were smaller than the lateral resolution of the NanoSIMS analysis (~ 100 nm). The lack of nanoparticle aggregates visible in the SE image demonstrates the absence of large (> 100 nm) aggregates of immunolabels. **(B)** The $^{197}\text{Au}^-$ ion image shows the distribution of the fluorinated colloidal gold immunolabels on the cell surface. **(C)** The $^{19}\text{F}^-$ ion image also revealed the locations of the fluorinated colloidal gold immunolabels on the cell surface. As expected, the $^{197}\text{Au}^-$ and $^{19}\text{F}^-$ secondary ions were co-localized in the image, indicating that both secondary ion species located the influenza hemagglutinin on the cell surface.

Figure 6.

Mosaic of overlapping NanoSIMS images that were acquired on immunolabeled and unlabeled (negative control) Clone 15 cells that contained metabolically incorporated ^{13}C -lipids. (A) Secondary electron (SE) images show the morphology of the surface of the immunolabeled Clone 15 cell. (B) The $^{19}\text{F}^-$ NanoSIMS images acquired in parallel with the secondary electron (A) and ^{13}C -enrichment (C) images reveal the location of influenza hemagglutinin in the membrane of the immunolabeled cell. (C) The ^{13}C -enrichment images show the distribution of ^{13}C -lipids in the plasma membrane of the immunolabeled Clone 15 cell. (D) Secondary electron images of the surface of the negative control cell were acquired in parallel with $^{19}\text{F}^-$ and ^{13}C -enrichment images. (E) Few non-specifically adsorbed immunolabels were present on the negative control Clone 15 cell. (F) The ^{13}C -enrichment images confirmed that ^{13}C -lipids were present in the plasma membrane of the negative control Clone 15 cell.

Table 1

Fraction of PFDT in the monolayers on gold nanoparticles that were modified with various ratios of MUDA:PFDT measured with XPS.

MUDA:PFDT ratio in reaction mixture	10:1	7:1 with delay [†]	1:1	1:10
Number of samples analyzed	4	6	6	4
Mean fraction of PFDT in monolayer $\pm$ 1 s.d.	0 $\pm$ 0	0.16 $\pm$ 0.11	0.22 $\pm$ 0.07	0.54 $\pm$ 0.10

[†] MUDA was added 2 min after the addition of PFDT (see text for details).