

Protein delivery with nanoscale precision

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Abstract

A novel assay of protein delivery to a surface with nanoscale precision was established. This was achieved by combining recent advancements in atomic force microscopy (AFM) and bioconjugation. We utilized a heterobifunctional photocleavable cross linker to functionalize an AFM tip with proteins. Upon irradiation, the proteins were released from the tip due to a photolytic reaction of the cross linker. These proteins bound tightly to their binding partners on a substrate. When tip functionalization is carefully controlled, proteins can be locally delivered to a desired area. Importantly, the result of protein delivery can be examined immediately by high-resolution imaging in the same area using the protein-free tip. Successful protein delivery was also confirmed by fluorescence imaging and was proved to be reproducible. The approach allows protein delivery and subsequent imaging to be performed in the same local area with the same AFM tip, thus opening up the possibility of monitoring protein functions in living cells in real time.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

The examination of living cells on the nanometre scale has attracted a lot of interest in recent years. This is because the elucidation of cellular functions at the molecular level can provide novel strategies for correcting cell dysfunction, thus opening up new avenues for drug design. Cell functions are usually mediated by the specific binding of a ligand to its membrane receptor, followed by the dynamic response of the receptor to initiate internal signal transduction pathways. Thus the investigation of cell function on the nanometre scale requires a tool that can allow not only the precise delivery of a protein to a specific location at a specific time, but also the visualization of the consequence of the protein delivery. In this paper, we report on our development of such a novel tool by combining recent advancements in atomic force microscopy (AFM) and novel bio-conjugation techniques.

AFM has become a powerful tool in biological research. The major advantages of AFM over other tools in biological studies are that no extensive sample preparation is required, and

surface features of various biological samples can be examined three-dimensionally under physiological conditions. High-resolution imaging has led to the study of the submolecular structures of proteins [1–7]. Besides topographical imaging, the ability of an AFM to quantify the interactions between molecules attached to the tip and molecules at the sample surface opens up an entirely new field for probing the specific binding force between a biomolecular pair [7–11]. More strikingly, AFM can be used for protein manipulation on the nanoscale. It was reported recently that a chemically modified AFM probe can mechanically uproot individual membrane proteins from a living cell [9, 12]. In these cases, AFM is used as a single-function tool, providing topographic imaging, force measurement, or protein manipulation. A bare tip was able to probe both mechanical properties and topographic characteristics of living cells [13–16]. It was also demonstrated that the stylus of an AFM probe can be used to dissect isolated DNA and proteins by applying a higher force on the AFM probe [17–19]. The result of dissection can be examined by subsequent imaging using the same bare tip. Although in this case AFM is used as a bifunctional tool, its role in surface manipulation is less constructive.

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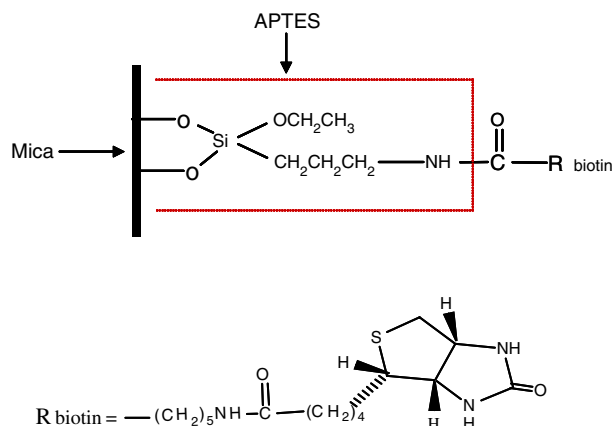
In this work, we established an assay that allows the AFM to accomplish two individual tasks sequentially: protein delivery and topographic imaging in the same local area in fluid media. Dip pen nanolithography (DPN) is a well established method for protein patterning at the nanometre scale [20–23]. It uses an AFM tip to deliver proteins to a surface via a solvent meniscus, which naturally forms in the ambient atmosphere. While DPN is successful in forming nanoscopic protein patterns, it cannot be used to deliver proteins in physiological media due to its dependence on the relative humidity and the solvent meniscus [22]. Since some protein molecules remain on the tip after the protein delivery, a separate tip is usually used to carry out the imaging of the patterned protein arrays. In our previous work, we synthesized a heterobifunctional photocleavable cross linker, succinic acid succinimidyl ester 5-thioxyloxy-2-nitrobenzyl ester (SSTN) [24]. SSTN has *N*-hydroxysuccinimidyl and disulfide groups at each end, and thus can covalently anchor protein molecules to a gold-coated AFM tip. In contrast to all the cross linkers that have been used in AFM tip modification to date, SSTN provides photocontrollable protein immobilization. We have reported protein delivery in air as an application of the SSTN cross linker [24]. Upon light irradiation, protein molecules anchored to the AFM tip can be released from the tip to the substrate via a photolytic reaction of SSTN. The protein-free tip can then be used to perform topographic imaging of the same local area. However, the condition of protein delivery in air does not mimic the living environment of proteins; hence, the results may not be applicable to the study of living cells. In addition, we observed that proteins delivered in air were not stable on the substrate, and were easily displaced by the AFM tip during imaging. In this work, we are reporting successful protein delivery under semi-physiological conditions. We chose the avidin–biotin system to prove our concepts. We have also demonstrated that the tip after protein delivery can be used to produce high-resolution images. This capability is critical to gathering information on the nanometre scale in living cells, and offers the possibility of monitoring ligand-induced receptor response in living cells in real time.

2. Experimental details

Materials

6-((biotinoyl)amino)hexanoic acid, succinimidyl ester (biotin-X-SE), was purchased from Molecular Probes (Eugene, OR). *N*-*N*, di-methyl formamide (DMF, 99.8%) and acetonitrile (HPLC gradient grade) were purchased from Acros Organics (New Jersey). Phosphate-buffered saline (PBS) with pH 7.4, avidin (A-9275, from egg white), green fluorescence protein (GFP), 3-aminopropyltriethoxysilane (APTES, 99%) and toluene (anhydrous, 99.8%) were purchased from Sigma-Aldrich. Ruby muscovite mica was purchased from Ted Pella. Ultrapure water (18.2 M Ω) was obtained with a filtration system (model: Nanopure Diamond, manufactured by Barnstead) and was used throughout the experiments.

APTES was dissolved in toluene to achieve a concentration of 4.30×10^{-4} M. The biotin-X-SE solution of 2.56×10^{-4} M was prepared by dissolving biotin-X-SE in DMF. Avidin was dissolved in a 0.01 M PBS solution



Scheme 1. Schematic illustration of mica surface modification resulting from APTES treatment followed by biotin attachment. The exposed biotin group provides high affinity to avidin.

to achieve a final concentration of 1.0×10^{-7} M. Newly synthesized succinic acid succinimidyl ester 5-thioxyloxy-2-nitrobenzyl ester (SSTN) [24] was dissolved in acetonitrile to achieve a final concentration of 3.60×10^{-4} M.

Mica substrate modification with biotin-X-SE

Freshly cleaved mica was immersed in the APTES solution for 2 h at room temperature under N₂ gas protection, allowing the silanization of a monolayer of APTES onto the mica substrate [25, 26]. The successful modification was proved by the increase of water contact angle (from 0° to 45°) [25, 26]. The modified substrate was ultra-sonicated in toluene for one minute and rinsed with toluene several times to remove the unbound residues from the surface. It was then immersed in the biotin-X-SE solution overnight at 4 °C under N₂ gas protection. The surface was ultra-sonicated in DMF for one minute and rinsed with DMF several times to remove the unbound biotin-X-SE residues from the surface to achieve a uniform monolayer. AFM images of the biotinylated mica showed a smooth surface with a surface roughness less than 0.5 nm in average. The water contact angle further increased (>90°) after the biotinylation. The structure of biotinylated mica surface is illustrated in scheme 1.

AFM tip functionalization with avidin

We functionalized avidin onto gold-coated AFM tips using the photocleavable cross linker SSTN [24]. Gold coating of AFM tips (3 nm titanium and 15 nm gold) was achieved by using a thermal evaporator (Denton Vacuum, Moorestown, NJ). The freshly coated tips were further cleaned via UV/ozone treatment for 30 min using a tip cleaner (BioForce Nanosciences). The cleaned cantilever was immersed into the SSTN solution for 12 h at 4 °C under N₂ gas protection, generating a monolayer of SSTN on the tip and the cantilever via Au–S linkage, as reported by others [27, 28] and as we routinely perform in the laboratory [7, 10, 24, 29]. The cantilever was then readily rinsed with acetonitrile to remove any loosely bound SSTN molecules. By incubating the modified tip with 1.0×10^{-7} M avidin in 0.01 M PBS solution

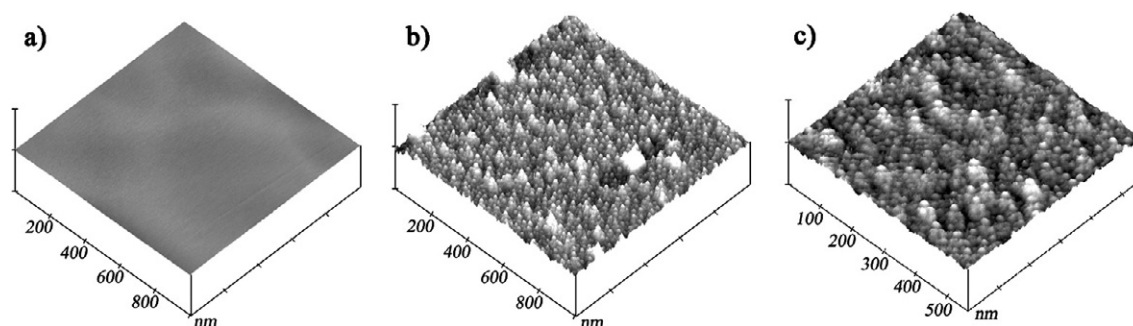


Figure 1. Three-dimensional height images collected on (a) a biotinylated mica surface and (b), (c) a biotinylated mica surface after incubation with an avidin solution of 1.0×10^{-7} M in PBS for 4 h. Image size: (a) $1 \times 1 \mu\text{m}^2$; (b) $1 \times 1 \mu\text{m}^2$; (c) $560 \times 560 \text{ nm}^2$. The Z data scale of (a)–(c) is 50 nm.

for 1 h, the hydroxysuccinimidyl group in SSTN reacted with the amine groups of lysines in avidin to form amide bonds [24], chemically linking avidin to the AFM tip surface. Any non-specifically bound avidin was extensively washed away with PBS buffer followed by ultrapure water. The avidin-functionalized tip was kept in ultrapure water until usage.

For local functionalization of avidin at the tip apex, we used an SSTN-modified AFM tip to approach a thin layer of protein solution on a mica surface. The approaching process was manually controlled using the positioner of the AFM set-up, and was carefully monitored via the optical microscope equipped with the AFM to allow the tip apex only to be in contact with the protein solution. After 1 h incubation, the tip was withdrawn and thoroughly rinsed in PBS and ultrapure water to remove any unbound residue.

Atomic force microscope

This study was carried out using a multimode Nanoscope IIIa AFM (Digital Instruments, Santa Barbara, CA), equipped with a J-type scanner. Protein delivery and imaging were performed using avidin-modified oxide-sharpened Si_3N_4 tips (triangular 100 N tip) in 0.01 M PBS solution in a fluid cell. Protein delivery was achieved by directing irradiation to an avidin-modified AFM tip while it was engaged and scanning at a pre-set area of $0.0001 \times 0.0001 \text{ nm}^2$ and a scan rate of 0.02 Hz (in practice, the tip was fixed in position and in contact with the substrate) on a biotinylated substrate. 30 min irradiation was effective to photocleave SSTN according to our previous characterization [24], releasing avidin from the AFM tip. The irradiation was carried out using a 60 W mercury lamp equipped with an IR filter and a 313 nm band-pass filter (dose rate $1 \mu\text{W cm}^{-2}$). To minimize the light penetration through the sample, we applied irradiation parallel to the sample surface. Thermal drift caused by irradiation alone was within 1%. The protein-free tip was used to scan at this local area to capture images for examining the delivered proteins. The images were acquired in the fluid-tapping mode at a thermal resonance frequency of 8–10 kHz. The scan rate was less than 1.00 Hz, and the number of samples was 512.

Fluorescence microscope

Fluorescence microscopy was conducted to confirm the protein functionalization on AFM tip when an FITC-labelled avidin

was employed. It was also used to examine protein delivery on biotinylated glass slides. Imaging was carried out using an Olympus IX-70 microscope equipped with a $40\times$ objective ($\text{NA} = 0.55$). In a typical epi-fluorescence set-up, a 100 W mercury lamp illuminated the functionalized AFM probe or protein-delivered glass slide. A fluorescence filter set (No 41018, Chroma Technology), consisting of an excitation filter, a dichroic beam splitter and a barrier filter, was used. Fluorescence images (at wavelength beyond 500 nm) were captured by a CCD camera (Diagnostic Instruments) and digitally enhanced. A confocal fluorescence spectrometer (Multi-View 400, Nanonics, Jerusalem) was used to *in situ* monitor the photocleavage of GFP anchored on an AFM tip via SSTN.

3. Results and discussion

3.1. Bulk protein delivery

Due to the strong specific interaction between biotin and avidin, surface-modified biotin (see scheme 1) can attract free avidin in solution phase and tightly bind it to the substrate. Figure 1(a) shows the image of a biotinylated mica surface. The successful surface modification is indicated by the increase in the water contact angle at the surface after biotinylation. The surface is smooth and featureless with a surface roughness of less than 0.5 nm. This implies that a densely packed monolayer of biotin was formed. The biotinylated surface was incubated with a 1.0×10^{-7} M avidin solution in 0.01 M PBS at room temperature for 30 min. Specific binding of avidin on this surface was confirmed by a separate experiment, in which FITC-labelled avidin was employed. Strong and uniform fluorescence, indicative of avidin distribution, was observed from the surface even after repeated rinsing with PBS solution followed by ultrapure water.

Figures 1(b) and (c) show the AFM images of the avidin-bound surface after thorough rinsing. In the $1 \times 1 \mu\text{m}^2$ image (figure 1(b)), we observed tiny spherical particles densely and uniformly distributed across the surface. The surface roughness has increased to 2.0 nm. The height of the particles measured with respect to defect areas is 2.5 nm in average, suggesting a monolayer of avidin. Occasionally large bright features appear on the surface, most likely corresponding to large clusters of avidin molecules physically adsorbed on top of the avidin monolayer.

In the higher-resolution image (figure 1(c)), the lateral dimension of an individual particle is determined to be 15 ± 4 nm in diameter. Considering the avidin tetramer's size of $5.6 \text{ nm} \times 5.0 \text{ nm} \times 4.0 \text{ nm}$, derived from x-ray crystallography [30], each individual particle may correspond to an aggregate of several avidin tetramers. The size of the particles measured at different areas of the same sample or on other samples similarly prepared by using different tips is constant, thus excluding the possibility of tip convolution artifacts. To examine the reason for the constant size of avidin aggregates, images were also collected from samples prepared at avidin concentrations of 10^{-8} and 10^{-9} M (data not shown). With the decrease of protein concentration, the average size of the protein particles decreased. At the 10^{-9} M concentration, particles with an average size of $5 \text{ nm} \times 6 \text{ nm}$ and a height of 2 nm were observed, corresponding to individual avidin tetramers loosely distributed on the surface. The results showed the clear dependence of the size of the protein particles on protein concentration. This clarifies that the protein aggregation in figure 1 was caused by the higher concentration of protein in the solution. However, even the 10^{-7} M avidin solution was too dilute to cause protein aggregation in the solution phase. We speculate that the enhanced surface concentration plays a key role. During the extended period of protein incubation with the substrate, proteins in solution interact favourably with the solid substrate, giving rise to significantly higher surface concentrations of avidin. Given the same incubation time, a higher solution concentration results in a higher surface concentration. A significantly high surface concentration, in combination with protein–protein interactions, provides the driving force for protein aggregation. Nevertheless, this process is different from the specific binding induced protein aggregation as we observed previously [6], in which constant sized protein aggregates were observed when receptor modified substrates were incubated with protein solutions of various concentrations. The size of the protein aggregates was governed by the ligand–receptor binding events. In the current study, when the protein concentration was the same, similar sized protein aggregates were observed regardless of the presence of biotin on the substrate, indicating that the protein aggregation is irrelevant to the avidin–biotin specific interaction. The size of the protein aggregates may be governed by the number of proteins available as well as by the protein–protein interactions.

According to the experimental results, free avidin in the fluid phase can be efficiently recruited by biotin on the substrate via the specific interaction. At sufficiently high surface concentrations, avidin molecules bind to the biotinylated surface as aggregates. These results provide the foundation for local protein delivery in fluid as delineated below.

3.2. Protein delivery from an AFM cantilever

We modified the entire AFM cantilever with avidin (using 1.0×10^{-7} M solution) via an SSTN cross linker. The successful tip modification was confirmed using fluorescence imaging of the modified cantilever with FITC-labelled avidin (figure 2). Such a modified tip was used to acquire AFM images of a biotinylated mica surface. Identical to the result shown in figure 1(a), the surface was flat and lacked any

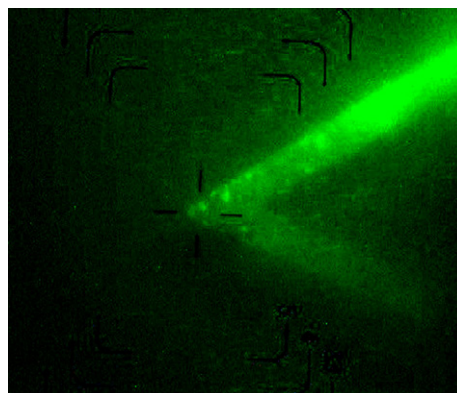


Figure 2. The fluorescence image of a triangular-shaped cantilever with FITC-labelled avidin covered the entire cantilever.

features (data not shown). While the tip was engaged and scanned a pre-set area of $0.0001 \times 0.0001 \text{ nm}^2$ with a scan rate of 0.02 Hz (in practice, the tip was fixed in position and in contact with the substrate), irradiation was applied to cleave the SSTN, thus releasing the avidin to the substrate. The same area of the surface was then imaged at $3 \times 3 \text{ }\mu\text{m}^2$ using the same protein-free tip as shown in figure 3(a). The image demonstrates a large cluster 324 nm long, 127 nm wide and 10 nm high at the centre (arrow pointed), surrounded by smaller particles with an average diameter of 13 ± 2 nm and an average height of 3.0 nm. After continuous scans at $1 \times 1 \text{ }\mu\text{m}^2$ for 10 min, we observed that the large cluster at the centre gradually disappeared while the number of small particles significantly increased.

Most likely, the large cluster in figure 3(a) corresponds to proteins delivered from the AFM tip apex while the small particles are proteins released from the cantilever. Since the tip apex was in contact with the biotinylated surface, as long as the proteins at the contact region were photocleaved from the tip apex, they tightly bound to the substrate due to the biotin–avidin specific interaction. Proteins photocleaved from the rest of the pyramid-shaped tip accumulated in the vicinity of the tip-projected area on the substrate, giving rise to a large protein cluster due to the availability of a great number of proteins in the local region. The height of the large cluster implies that the cluster may contain a layer of specifically bound avidin and additional layers of physically adsorbed avidin. The physically adsorbed proteins are relatively unstable. In fact, when the tip scanned continuously, the large cluster was perturbed by the tip, and the proteins gradually detached from the cluster as small aggregates. The aggregates scattered in the scan area and bound to biotins on the substrate. Proteins that were photocleaved from the rest of the cantilever could diffuse in the fluid and bind to the substrate via specific interaction as small aggregates. Analogous results were reproduced in multiple samples. The small aggregates had a size similar to those observed in bulk protein delivery, resulting from enhanced surface concentration and protein–protein interaction. When the tip scanned this area further, no re-distribution of the protein aggregates was observed, due to the strong avidin–biotin binding that held the proteins in position. This result also indicates that the delivered avidin maintained its biological

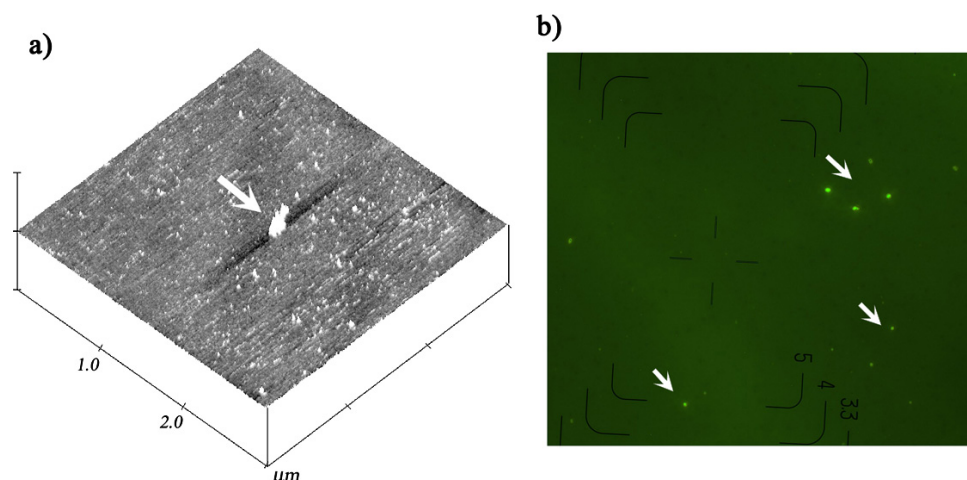


Figure 3. (a) A $3 \times 3 \mu\text{m}^2$ height image showing a protein cluster (arrow pointed) together with small protein aggregates delivered from an avidin-modified cantilever to a biotinylated mica surface. The Z data scale is 50 nm. (b) Fluorescence image of large protein particles (arrow pointed) and small aggregates of FITC-labelled avidin delivered from a similarly modified cantilever.

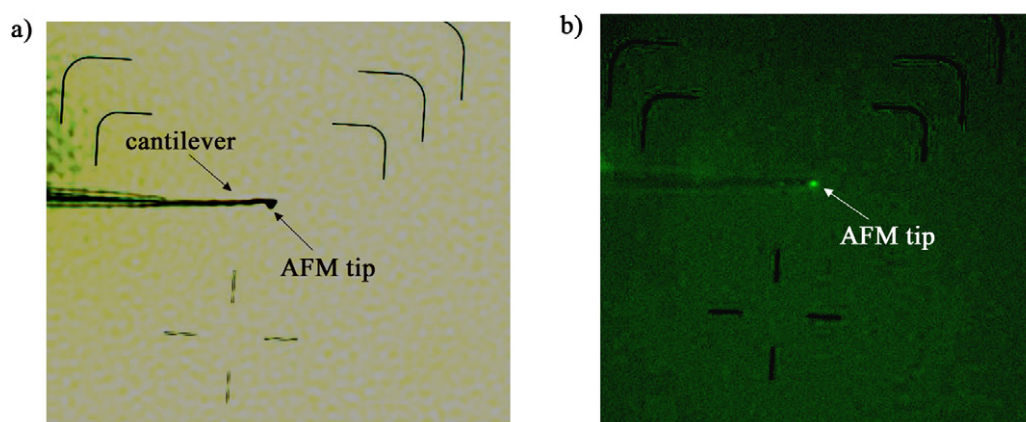


Figure 4. Optical (a) and fluorescence (b) images of the tip and the cantilever with FITC-labelled avidin locally modified at the tip apex.

activity, consistent with our previous report that the irradiation did not alter the avidin–biotin binding affinity [24].

To prove that the observed particles in the AFM images were indeed avidin photocleaved from the AFM cantilever, we performed fluorescence imaging of a biotinylated surface after FITC-labelled avidin molecules were delivered from an AFM cantilever in fluid in a similar way. Figure 3(b) illustrates the fluorescence image of such a surface, on which avidin aggregates are evident. Different from the single large protein cluster observed on the AFM image (figure 3(a), arrow pointed), several large protein particles (figure 3(b), arrow pointed) together with small protein aggregates were observed on the surface. Since the fluorescence image was not collected immediately after the protein delivery, as we did in the AFM study, the multiple large protein particles might result from the initial collapse and diffusion of the type of large single protein cluster seen in figure 3(a), which is unstable in the solution phase due to the non-specific binding nature of the majority of the proteins in the cluster. Consistent with the results of the AFM study, proteins photodelivered from the cantilever were bound to the biotinylated surface as small avidin aggregates and scattered in a broad area.

Our results indicate that when an entire cantilever is fully covered with avidin, the proteins can be delivered to the substrate with random distribution, as shown in figure 3. Proteins delivered right from the tip apex form a large cluster, which is unstable on the substrate because the majority of the proteins involved in the cluster are non-specifically bound species. To achieve precise protein delivery, we improved the system.

3.3. Local protein delivery

We immobilized the proteins exclusively at the tip apex, as described in the experimental section. The success of local tip modification was confirmed by examining the fluorescence image of a tip modified with FITC-labelled avidin. Figure 4 shows optical (figure 4(a)) and fluorescence (figure 4(b)) images of the AFM tip on its cantilever in the fixed tip position. Fluorescence was detected exclusively at the nominal tip apex.

As illustrated in figure 5(a), we positioned the tip on a surface of biotinylated mica. Irradiation was applied to the cantilever for half an hour while the tip was engaged. When the irradiation was terminated, the same area of the mica surface

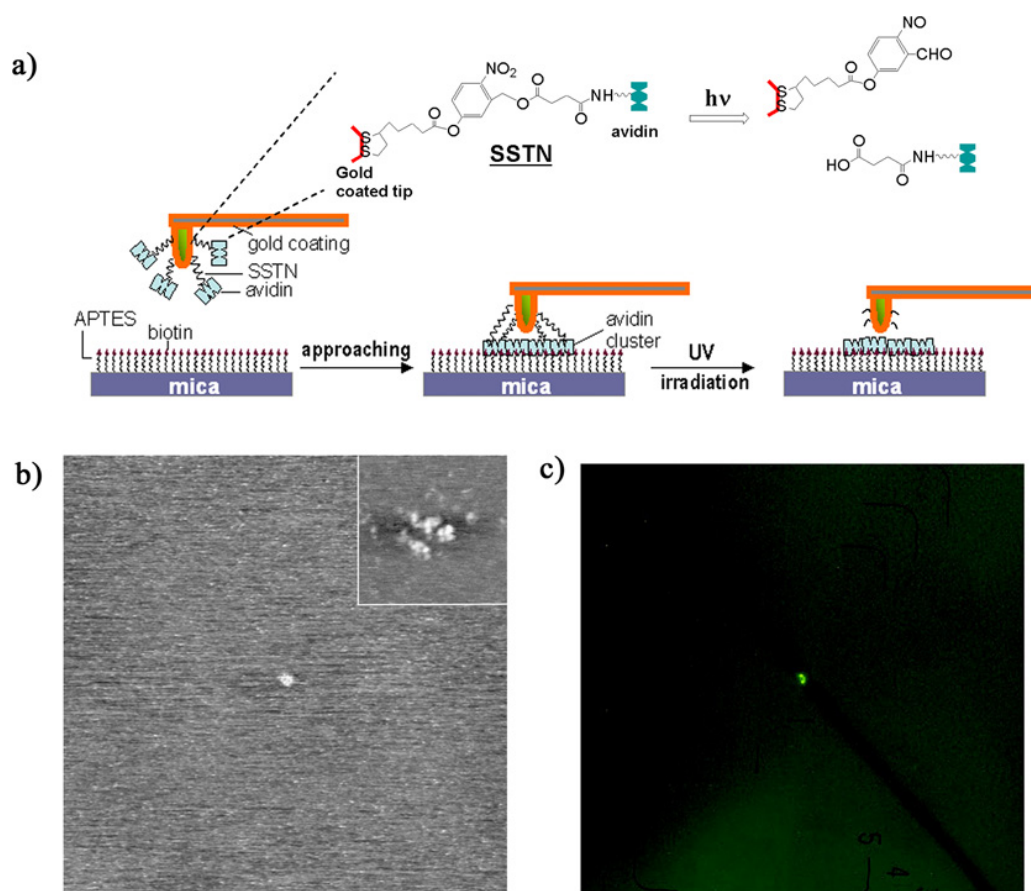


Figure 5. (a) Scheme of local protein delivery. (b) Height image of a biotinylated mica surface after local protein delivery, image size $3 \times 3 \mu\text{m}^2$, inset $400 \times 400 \text{ nm}^2$. (c) Fluorescence image of a biotinylated mica surface after local delivery of FITC-labelled avidin.

was imaged by the same protein-free tip. Figure 5(b) shows a $3 \times 3 \mu\text{m}^2$ AFM image. A single protein cluster $100 \pm 4 \text{ nm}$ long, $90 \pm 2 \text{ nm}$ wide and $2.5 \pm 0.2 \text{ nm}$ high appeared at the centre of the surface as a result of protein delivery from the tip apex. Thus we can infer that an area of about $100 \times 90 \text{ nm}^2$ at the tip apex was functionalized by a layer of avidin. When the tip was engaged and in contact with the biotinylated substrate, avidin on the tip specifically bound to biotins on the substrate. Upon photocleavage of SSTN, the avidin tetramers were released from the tip and delivered to the substrate. The protein-free tip can be used to collect images as efficiently as a bare tip. The inset in figure 5(b) is a $400 \text{ nm} \times 400 \text{ nm}$ image illustrating the details of the cluster. The cluster is composed of multiple protein aggregates with an average diameter of $12 \pm 2 \text{ nm}$ and an average height of $2.5 \pm 0.2 \text{ nm}$. Notice that the height of the cluster indicates that avidin was specifically bound to the substrate as a monolayer. In contrast to the cluster in figure 3(a), the protein cluster in figure 5(b) was quite stable and could sustain continuous scans without changing the cluster configuration. The result proved to be reproducible. This implies that the strong specific interaction can tightly hold avidin that is locally delivered from the tip to the substrate.

Local protein delivery was also confirmed using fluorescence imaging (figure 5(c)). Consistent with the AFM image in figure 5(b), we found a singular fluorescent spot right beneath the AFM tip on the entire sample surface, indicating precise control of protein delivery.

A control experiment was carried out using an AFM tip similarly modified with BSA using SSTN. Since BSA does not bind to biotin on the surface, the photocleaved BSA freely diffused in the fluid phase without attaching to the substrate. Thus AFM revealed a featureless mica surface even after irradiation was applied under the same condition. The experiment was also repeated with the BSA modification on the entire AFM cantilever. Similar results were achieved. These further confirm that the particles observed in figures 3 and 5 were the results of avidin delivery.

As shown in figure 5(a), the protein aggregates can be precisely positioned on a desired surface. The precision of protein delivery relies on the precision of the lateral control of the AFM, which can be reproducibly achieved on the nanometre scale. With the present experimental design, the delivered proteins were confined in a range of $100 \times 90 \text{ nm}^2$. When a sharper tip is used and the tip modification is further controlled by limiting the contact area between the tip and the thin layer of avidin solution, as depicted in section 2, we expect to deliver fewer protein aggregates in a more focused area. The number of delivered proteins can be further reduced by diluting the concentration of SSTN cross linker with another thiol-derivative that is unable to cross link proteins during tip functionalization. The work is under way. Since it allows control of protein delivery without the need for surface patterning, this method provides unique value when processing protein chips.

We also demonstrated that the same tip can be used after protein delivery to image the same surface area at a resolution as high as that achieved by a bare tip. Thus, the use of a photocleavable cross linker allows the subsequent accomplishment of two tasks in the same local region: (1) protein delivery, and (2) characterization of the delivered proteins. On a model surface like the one we chose for this work, biotins covered the entire substrate, providing homogeneous binding sites for the delivered avidin. When the avidin aggregates were delivered, each avidin tetramer in the aggregate bound firmly to the surface via specific bonds, and each protein aggregate remained intact as a unit on the surface. This occurred because the model surface is relatively rigid and the avidin–biotin complex lacks mobility due to the chemical bonding of biotins on the substrate. The situation may be entirely different if we apply this approach to delivering ligands to a cell membrane. A cell membrane contains heterogeneously distributed receptors at a much lower density. Guided by the specific interaction via force mapping [7, 10, 31] using the ligand functionalized tip, cell membrane receptors can be registered; when a ligand is dosed in the receptor-rich area as a protein cluster, receptors may move laterally and dynamically associate with each other; the local cell response to the ligand–receptor binding can be *in situ* monitored in a living cell on the nanometre scale. To optimize the irradiation condition for living cell studies, we conducted experiments of monitoring the photocleavage process of green fluorescence protein (GFP) anchored on an AFM tip via SSTN by using a confocal fluorescence spectrometer. The irradiation was carried out under the same conditions. We observed that the fluorescence intensity of the tip decreased with the irradiation time, and declined to the background level within three minutes. Steady-state fluorescence study of pure GFP indicated that GFP fluorescence intensity remained unchanged upon three minutes irradiation, indicating that no protein denaturation nor bleaching occurred. Thus we conclude that the fluorescence intensity decrease of the AFM tip was due to the removal of GFP from the tip, and virtually all cross linker molecules were cleaved away within three minutes. The same doses of irradiation were applied to living mouse embryonic fibre blast cells, and no observable difference in cell proliferation rate (via cell counting) was found between the irradiated cells and the control sample cells (without irradiation). Thus the methodology derived in this study can be generally adapted in any ‘nanobiological’ research.

4. Conclusion

We have demonstrated that precise protein delivery to a surface with a high-affinity binding partner can be achieved in aqueous media. This can be carried out using an AFM cantilever that is locally modified with protein at the nominal tip apex via a photolabile cross linker. We positioned the modified AFM tip in the desired area. When proteins on the tip were in contact with their binding partner on the substrate, the proteins were released from the tip via a photolytic reaction of the cross linker and delivered to the desired area on the substrate

with nanoscale precision. Successful protein delivery was confirmed using both AFM imaging and fluorescence imaging. This approach also allows protein delivery and subsequent imaging to be performed with the same AFM tip, thus opening up the possibility of monitoring protein functions in living cells in real time.

Acknowledgments

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