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High-throughput Enzyme Nanopatterning

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Abstract

High-throughput and large-scale patterning of enzymes with sub-10-nm resolution, the size range of individual protein molecules, is crucial for propelling the advancement in a variety of areas, from the development of chip-based biomolecular nano-devices to the molecular-level studies of cell biology. Despite recent developments in bio-nanofabrication technology, combining 10-nm resolution with high-throughput and large-scale patterning of enzymes is still an open challenge. Here, we demonstrate a high resolution and high-throughput patterning method to generate enzyme nanopatterns with sub-10 nm resolution by using thermochemical scanning probe lithography (tc-SPL). First, tc-SPL is used to generate amine patterns on a methacrylate copolymer film. Thermolysin enzymes functionalized with sulfonate-containing fluorescent labels (Alexa-488) are then directly immobilized onto the amine patterns through electrostatic interaction. Enzyme patterns with sub-10 nm line width are obtained as evidenced by atomic force microscopy (AFM) and fluorescence microscopy. Moreover, we demonstrate large-scale and high throughput (0.13×0.1 mm^2 at a throughput of 5.2×10^4 μm^2/h) patterning of enzymes incorporating 10-nm detailed pattern features. This straightforward and high-throughput method of fabricating enzyme nanopatterns will have a significant impact on future bio-nanotechnology applications and molecular-level biological studies. By scaling up using parallel probes, tc-SPL is promising for implementation to scale up the fabrication of nano-biodevices.

1. Introduction

Enzymes are a class of biomolecules which are known to catalyze a variety of biochemical reactions with great specificity under mild conditions[1] and have been exploited for decades in various fields, such as biological studies, biomedicine, bio-nanotechnology and in the food industry. Enzyme immobilization on surface patterns with predefined configurations and spatial control has garnered significant interest and shows significant importance in many biological and biotechnological applications, such as biosensors[2, 3], enzyme-assisted lithography[4], bio-

nanoreactors[5, 6], as well as in the systematic studies of biomolecule interactions[7]. Enzyme immobilized on solid supports also constitute functional surfaces which may exhibit the advantages of improved enzyme stability and reusability after multiple exposures to reaction mixtures.

To fully realize the potential of enzymes for applications in bio-nanotechnology, the ability to generate enzyme surface patterns at the nanoscale is critical. Since miniaturized size and greater detection sensitivity are desired for future biosensors, highly defined enzyme nanopatterns and single-enzyme positioning capability could significantly facilitate the fabrication of biosensors with densely packed arrays[4, 8, 9]. Also, with the capability of catalyzing the degradation of certain macromolecules, enzyme nanopatterns can also be used as a lithography tools to generate templates for nanoreactors, photonic structures and electrical circuits[8, 10, 11]. Furthermore, patterning enzymes in quasi 2D, soft polymer landscapes and using feature sizes that are in the same (<10 nm) range as these enzymes, is advantageous as it allows a more precise engineering of the enzymes' microenvironment, potentially allowing for designing this environment to more closely mimic the biological context and thereby to enhance enzyme function and stability.

Fabrication of enzyme surface patterns relies on advanced patterning techniques which were initially developed for the semiconductor industry and now have been translated to a variety of fields, such as biomedicine and bio-nanotechnology. These patterns have been successfully generated using a variety of techniques, including micro-contacting printing[11-13], inkjet printing[14] and optical lithography[15-17] which can readily generate large-scale patterns but lack the capability of patterning at the nanoscale. Electron beam lithography (EBL)[18-21], dip pen nanolithography (DPN)[10, 22, 23], nanoimprint lithography (NIL)[24], scanning probe lithography (SPL)[25-28] and bottom-up self-assembly[29-32] can generate patterns at the nanometer scale and several works report enzyme patterns with sub-100 nm resolution using these methods. However, it is still challenging to fabricate enzyme patterns with sub-10 nm resolution using a high-throughput top-down method in a facile and effective manner, avoiding complex and multi-step fabrications. Only few works[22, 29] reported enzyme immobilization onto patterns with 10 nm resolution. For example, Chai *et al* reported the only top-down approach that generated an array of 10-nm Au nanoparticles with avidin-horseradish peroxidase attached using DPN[22]. However, multiple steps of fabrication are required to produce the 10-nm Au dots and protein immobilization is needed for finally immobilizing enzymes. As a bottom-up approach, self-assembly has been utilized to achieve enzyme patterns of sub-10 nm resolution[29]. However, the lack of specific control over enzyme density, pattern configuration and pattern topography hinders its further implementation[33]. Additionally, a direct proof of enzyme immobilization on the patterns by AFM or fluorescence microscopy is missing. Moreover, for future industrial-scale applications, one of the most important criteria is large-scale manufacturing using high-throughput and cost-effective fabrication processes. Therefore, to fulfill the potential of enzyme nanopatterns for large-scale bio-nanotechnology applications, it is important to develop a fabrication process that can achieve high-throughput and large-scale enzyme patterning together with ultrahigh resolution.

In our previous works[34-37], protein and DNA patterns were demonstrated by using a home-built thermochemical SPL (tc-SPL) system, where the heated scanning probe induced the local amine deprotection on a synthesized methacrylate copolymer film followed by the immobilization of the proteins and DNAs through appropriate linker chemistry. Although those works demonstrated the great versatility of this method to pattern biomolecules, the smallest object achieved was only around 30 nm in lateral dimension. Also, they required intermediate linkers to bridge the proteins or DNAs to the amine patterns with extra steps and increased cost. Furthermore, due to the limited throughput, no large-scale patterning was demonstrated.

Here, we report a SPL method for generating robust enzyme nanopatterns with sub-10 nm resolution and with large-scale and high-throughput patterning capability (0.13×0.1 mm^2 at a throughput of 5.2×10^4 μm^2/h). This is enabled by using a tc-SPL system (see experimental part) featuring simultaneous patterning and *in-situ* imaging, and the immobilization of enzymes onto the amine patterns through electrostatic interactions between negatively charged enzymes and positively charged amine patterns (Fig. 1c). This electrostatic immobilization approach greatly simplifies the fabrication process and opens up a door for generating nanopatterns of charged biomolecules using our fabrication method. This method of generating high-throughput and high-resolution enzyme nanopatterns may significantly impact the future fabrication of highly sensitive biosensors and the studies of cell behaviors on bio-surfaces.

2. Experimental

Materials

The polymer used in this work is a non-commercial diblock polymethacrylate copolymer, poly((tetrahydropyran-2-ylN-(2-methacryloxyethyl)carbamate)-co-(methyl 4-(3-methacryloylox-ypropoxy)cinnamate)) with 1:4 ratio of cinnamate to carbamate, which was synthesized using uncontrolled free-radical polymerization according to previous reports[38]. The polymer contains thermally labile tetrahydropyran (THP) carbamate groups. It deprotects and exposes the primary amines upon heating at the deprotection temperature ($T_0 \sim 150$°C)[34, 35, 37]. The fluorescence fluorophore, Alexa-488 NHS ester was purchased from ThermoFisher and dissolved in dimethyl sulfoxide (DMSO) to obtain a 2 mM stock solution. It was further diluted by water to 100 nM for incubating patterned films. The enzyme, thermolysin, was purchased from *Bacillus Thermoproteolytics Rokko* and conjugated with Alexa-488 NHS ester fluorophore for enzyme immobilization and optical detection.

Polymer film preparation

The polymers were dissolved in chloroform to obtain solution with the concentration of 2.5 mg/mL followed by the filtration with 0.45 μm PTFE filters (Thermofisher). The polymer solution was

spin coated onto Si substrate (1500 rpm, 60s) followed by a quick baking (50°C, 1 min) to remove the residual solvent. The resulting film thickness ranges from 20 to 30 nm.

Synthesis of Alexa-488 thermolysin conjugate

4 mg of thermolysin was dissolved in 3 mL water (pH is adjusted to 8) followed by adding 60 µL Alexa-NHS ester solution with a concentration of 2 mM in DMSO. The solution was then shaken for two hours. After dialysis of the solution to wash off unreacted dye (dialysis membrane with Molecular Weight cut off = 3500 Dalton), the remaining solution was lyophilized to obtain Alexa-488 conjugated thermolysin (Alexa-thermolysin). The product formation was confirmed by mass-spectrometry (MALDI).

tc-SPL and pattern fabrication

Thermal Patterning of the polymer films was conducted in ambient environment using a commercial tc-SPL system (NanoFrazor, SwissLitho AG, Switzerland). It is equipped with a silicon thermal cantilever comprising a resistive microheater for thermal patterning and a separate resistor used as a thermal sensor for topography imaging. The temperature of the microheater on the cantilever is calibrated by the NanoFrazor software by fitting the knee point in the current-voltage curve with the theoretical values for doped silicon[39]. During the patterning, the cantilever is in contact with the sample surface. The cantilever is bent down by the electrostatic force between the cantilever and Si substrate, and the bimorph bending due to the temperature gradient in the cantilever[40]. Another major feature of this lithography system is the capability of simultaneous patterning and in-situ imaging. After each patterning line, the thermal reading sensor immediately maps the topography of the patterned structure when retracing back in contact mode. This also enables the closed feedback loop correction for 3D patterning.

In this work, patterning was performed with a pixel dwell time 140 µs and a pixel pitch of 30 nm for patterns in Fig. 2 to Fig. 4. For the NYU Tandon pattern in Fig. 5, pixel dwell time and pixel pitch are 50 µs and 50 nm (1mm/s), respectively. In detail, the NanoFrazor system was operated in a pulsed heating mode by which the probe was only heated shortly before and in contact with samples. This operating mode extends its lifetime. The typical setting of heating pulse and electrostatic force pulse used in this work were 120 µs and 100 µs, respectively (for pattern in Fig. 5, 30 µs and 15 µs were used). The patterning temperature was typical above the amine deprotection temperature (T_0 ~ 150°C). It should be noted that the patterning temperature at the probe-sample interface cannot be directly measured due to a reduced heat transport from microheater to the sample surface. To estimate the contact temperature at the probe-sample interface, a heating efficiency of 0.24 between the temperature of the microheater and the probe-sample interface was derived and shown to follow the equation $T_{contact} = 0.24(T_{heater} - T_{RT}) + T_{RT}$.[41-43]

Fluorophore and Enzyme Immobilization

Alexa 488 was immobilized onto the amine patterns by incubating the polymer film with 100 nM Alexa 488 NHS ester water solution for 45 minutes. After incubation, the surface was rinsed with Milli-Q water (ultrapure and filtered) and phosphate buffer solution (PBS), and then dried with N_2. Thermolysin enzyme was immobilized by incubating the polymer film in 30 µL of 1 mg/mL Alexa 488-thermolysin conjugate solution in water for one hour. The above-mentioned washing and drying procedures were performed.

Pattern characterization

Fluorophore-labelled patterns were imaged using laser scanning confocal fluorescence microscopy (Zeiss LSM 880 Airyscan) with excitation laser wavelength λ_{exc} = 488 nm and emission detection window λ_{em} = 510-600 nm. The fluorescence images were processed using ImageJ and the optical contrast was tuned for better visibility. All AFM measurements were conducted on a Bruker MultiMode 8 AFM. Gwyddion software was used to Flatten and adjust the z-scale of AFM images.

3. Results & Discussion

Amine patterns and enzyme immobilization

The critical step for achieving enzyme patterns is to fabricate active amine patterns. As reported in our previous works[34, 35, 37], the heated probe induces local deprotection of amines on the surface of the polymer films. Fig. 2a shows several square patterns generated by the tc-SPL system with different temperatures (141°C to 191 °C). To confirm the deprotection of primary amines on the patterned surface, a green-emitting fluorophore, Alexa-488 NHS ester was used to label these amines. From Fig. 2b, the enhanced emission intensity from the square patterns confirms that amine groups are exposed on the patterned surface. It is shown that different emission intensity is obtained from different squares. This is due to the fact that the amine deprotection is a thermally-driven reaction and thereof the final amine concentration strongly depends on the patterning temperature. The patterns shown here are only used to confirm the presence of amines. Detailed discussion on the temperature dependence of the amine deprotection can be found in previous work[37].

Following from the successful fabrication of active amine patterns, we seek to immobilize enzymes onto amine patterns through a simple and effective approach. This is based on the specific electrostatic interactions between positively charged amines and negatively charged Alexa-488 sulfonate groups. Thus, thermolysin enzyme was modified by covalently conjugating with Alexa-488 which contains sulfonate groups (see experimental part) such that it can strongly adhere onto positively charged amine surfaces created by tc-SPL. In this way, the enzyme patterns can be also

easily imaged by using fluorescence microscopy. To test our hypothesis, both AFM and fluorescence microscopy were used to characterize the patterns after incubation with thermolysin enzyme solution and results are shown in Fig. 3. Here, an array of 1×1 μm^2 square-patterns were generated by tc-SPL with temperature above amine deprotection temperature. The AFM topography images of a single square pattern before and after incubation (Fig. 3 a and b) shows that after incubation, the pattern is filled up with the immobilized proteins. The fluorescence image of this array of square patterns shows the enhanced emission intensity from the square patterns (Fig. 3c), which confirms the selective immobilization of Alexa-thermolysin molecules onto the patterns. The cross-sectional profiles (Fig. 3d) shows that height difference of the pattern before and after enzyme immobilization is around 6 nm. This indicates the immobilization of single layer of thermolysin enzyme molecules onto the pattern (thermolysin enzyme size ~ 5.75 nm, see Supplementary Information for the size characterization of the thermolysin molecules).

Sub-10 nm enzyme patterning with single-enzyme resolution

With thermolysin enzymes successfully immobilized onto the micro-scale patterns, we investigate the limit of the patterning resolution in x, y. Since features smaller than 100 nm cannot be easily imaged by fluorescence microscopy, in order to probe and visualize the enzyme immobilization onto such small features, we patterned a 1×5 μm^2 rectangular area consisting of multiple single-line patterns with amines exposed on the surface (Fig. 4a). From the cross-sectional profile (Fig. 4c, purple curve), it is shown that each individual line has a full width at half maximum (FWHM) of 9 nm comparable to the size of a single enzyme molecule. This ultrahigh resolution benefits from the sharp temperature gradient produced by the ultra-sharp tip integrated within this lithography system[44]. After the enzyme immobilization, similarly, those patterned lines are filled up by the enzyme molecules (Fig. 4b and red cross-sectional profile in Fig. 4c). From the fluorescence emission of this area (Fig. 4d), the selective immobilization of enzymes onto these single-line patterns is clearly shown, which also confirms the patterning of single-enzyme lines. Using this method, sub-10 nm enzyme patterning with remarkable control of pattern position and configuration can be readily achieved, which may greatly benefit the scaling of future biosensor and bio-nanoreactors.

Combination of large-scale patterning of enzymes and high resolution

After demonstrating the smallest feature of enzyme patterning by this new tc-SPL system, we explore its capability for generating large-scale enzyme patterns. The logo for NYU Tandon School of Engineering was selected for the large-scale enzyme patterning experiments. Fig. 5a shows the fluorescence image of a 0.13×0.1 mm^2 thermolysin enzyme pattern of NYU Tandon logo generated in 15 min (5.2×10^4 $\mu m^2/h$). This straightforward and large-scale patterning is enabled by the high-throughput capability of this system (up to 5.2×10^4 $\mu m^2/h$ per single probe). To date, this is the largest enzyme pattern achieved using scanning probe lithography techniques.

Beside the high throughput, simultaneously *in-situ* thermal imaging empowers this lithography system to perform real-time metrology and monitor the patterning process, which greatly enhances the fabrication efficiency. This is a significant advantage over other mainstream lithography techniques, such as optical lithography, EBL and DPN. Figure 5b shows an example of *in-situ* thermal topography image of "NY" obtained simultaneously during the patterning. Furthermore, when we zoom in the patterned region, it is shown that the smallest component of this large logo pattern, i.e. single patterning line has FWHM around 10 nm. Although this hasn't reached the resolution limit of this technique, it is still among the top list of patterning resolution of any other lithography technique. This demonstrates the capability of generating large-scale enzyme patterns with high resolution details. When scaled up by using multiple parallel probes, this method of patterning enzymes can have remarkable potential for fabricating industrial-scale biochips and will also facilitate the studies of cell behaviors on bio-surfaces.

4. Conclusion

In this contribution, we present a high-throughput and high resolution patterning method to generate enzyme patterns using thermal scanning probe lithography. With amine patterns first generated by tc-SPL, thermolysin enzymes functionalized with sulfonate group were directly immobilized onto positively-charged amine patterns under aqueous condition through electrostatic interaction without any intermediate linker, improving greatly the efficiency of the fabrication process. We demonstrate that, by using our new tc-SPL system, sub-10 nm (single-enzyme level) enzyme patterns can be achieved. In comparison to the work achieving 10 nm enzyme patterns using top-down dip pen nanolithography[22], our method is more cost-effective and requires a significantly easier fabrication process. Together with the advantage over bottom-up self-assembly methods of controlling the spatial arrangement and configuration of the patterns, our method introduces a facile and highly effective way of generating arbitrary enzyme patterns with single-enzyme resolution (sub-10 nm). We also demonstrate the high-throughput (5.2×10^4 $\mu m^2/h$) and large-scale (0.13×0.1 mm^2) patterning of enzymes combined with 10-nm pattern details using a single probe. This is shown to be very promising for future fabrication of industrial-scale biochips and provides an alternative method to generate bio-patterned surfaces for studies of cell behaviors.

Conflicts of interest

There are no conflicts to declare.

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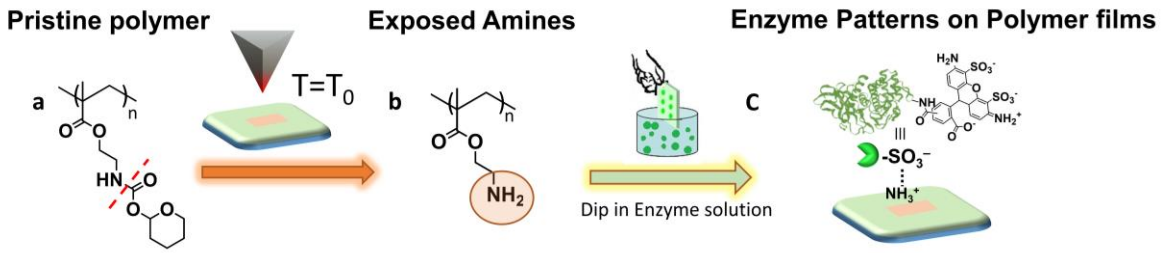

Fig. 1. Schematic illustration of the general process of generating enzyme patterns by tc-SPL. (a-b) Generating amine patterns by locally deprotection of the amine groups using a heated probe. (b-c) Enzyme immobilization onto amine patterns through electrostatic interactions.

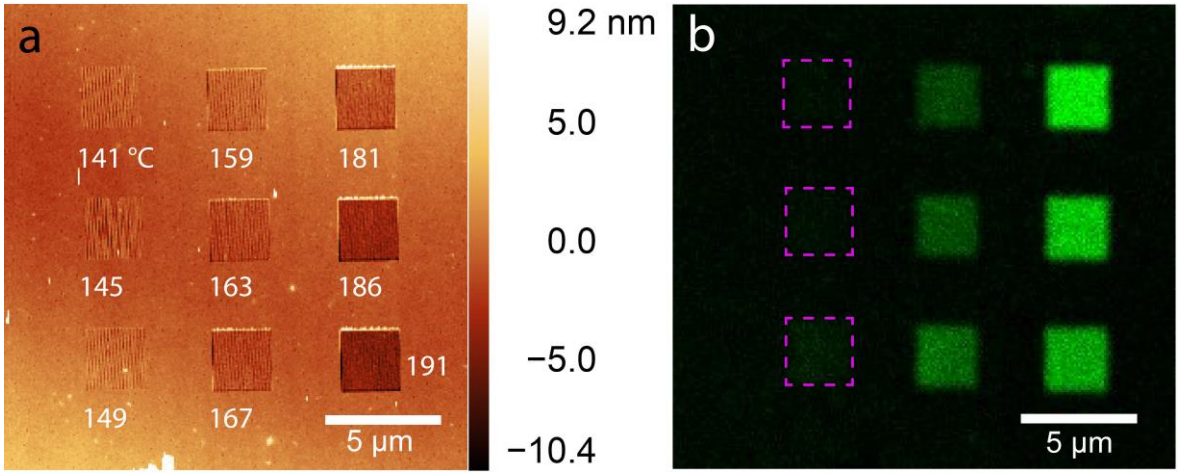

Fig. 2 Amine patterns generated by tc-SPL. (a) AFM topography image of square patterns using different temperatures. (b) Corresponding fluorescence image of the amine patterns labelled by Alexa-488 NHS ester.

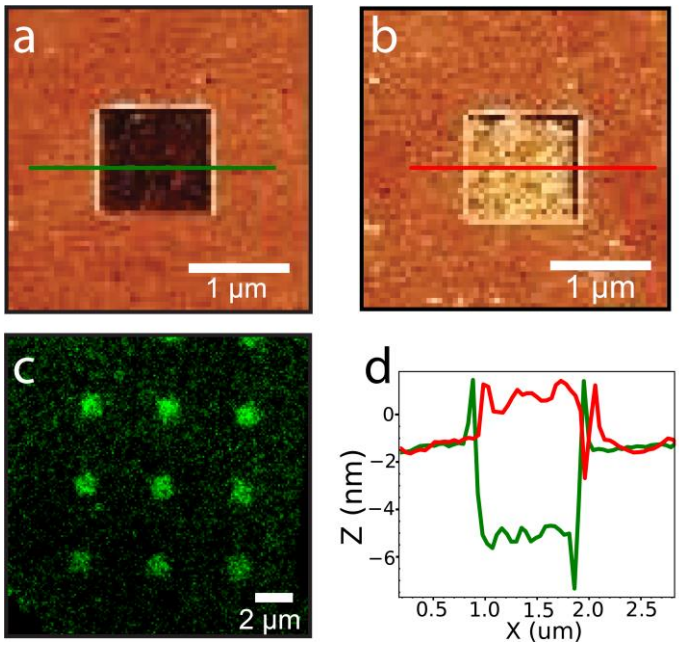

Fig. 3 1×1 μm^2 square amine patterns filled with electrostatically immobilized thermolysin. (a-b) AFM topography images collected before (a) and after (b) thermolysin immobilization (z-scale is 16 nm). (c) Confocal fluorescence images of an array of square patterns. (d) Cross-sectional profiles from green line in (a) and red line in (b).

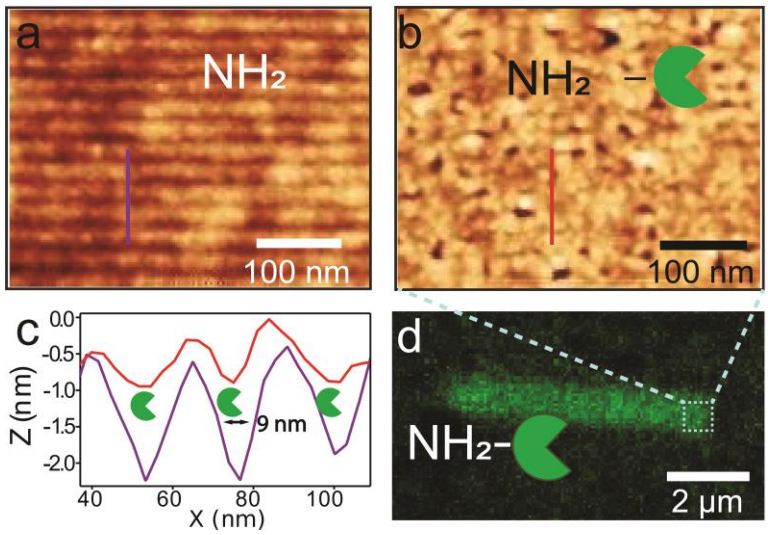

Fig. 4 Sub-10 nm thermolysin enzyme patterns. (a, b) AFM topography images of the single-line amine patterns before (a) and after (b) thermolysin immobilization (z-scale is 10 nm). (c) Cross-sectional profile from purple line in (a) and red line in (b), showing 9 nm FWHM. (d) Fluorescence image of the entire 1×5 μm^2 rectangular pattern consisting of closely-packed single lines with thermolysin enzyme immobilized.

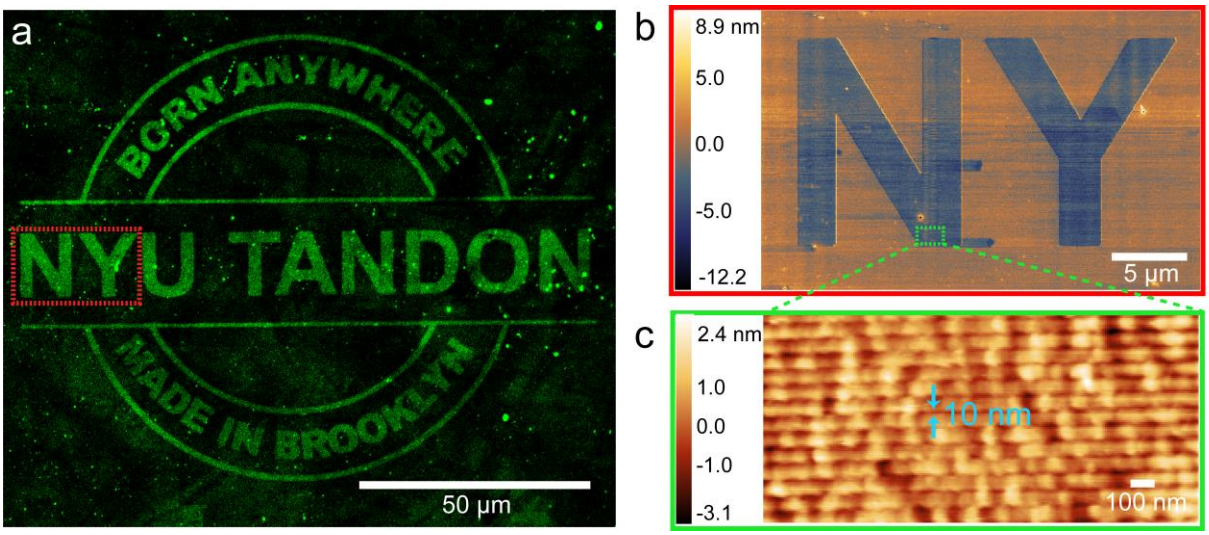

Fig. 5 Combination of high resolution and large-scale patterning of enzymes. (a) 0.13 × 0.1 mm^2 enzyme pattern of NYU Tandon School of Engineering logo. (b) Thermal topography image of patterned "NY", as shown in panel (a), obtained during the simultaneous tc-SPL patterning and in-situ thermal imaging. (c) AFM topography image of the zoomed-in area in (b), showing high resolution single-line patterning of 10 nm FWHM. Permission to use logo was granted by the New York University Trademark Licensing.