Anodic Porous Alumina Nanoarrays for obtaining gene/protein based microarrays

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Abstract

We hereby report the protocol used for obtaining gene/protein based microarrays by spotting biomolecules onto a matrix of anodic porous alumina (APA) that can be used as a laboratory-on-a-chip. APA, characterized by a highly ordered hexagonal pores arrangement, can be, indeed, used and exploited as a resistant mechanical support/scaffold for biological procedures and manipolations such as gene/protein expression. The present protocol was formulated starting from an amorphous aluminium microstructure surface and represents the successful conjunction and coupling of three technologies:1) APA obtained via aluminium anodization and electrochemical route, 2) label-free Nucleic Acid Programmable Protein Arrays (NAPPA), 3) LB nanotemplate and piezoelectric inkjet printing of SNAP Genes utilizing bacterial cell free expression system, capable to make unique nanoarrays contributing to the solution of the numerous problems open in structural and functional proteomics for Cancer control.

Introduction

In recent years, geometry and architecture of materials fabricated at the nano- and micron-scales, with regular shape and capacitive property, have fascinated researchers in physics as well in the field of proteomics science. Nano-rods, nano-wires, nano-particles and nano-cage shaped materials are only some of the structures with such properties to be able to confine liquids with peculiar physical characteristics[1-4]. Scaffolds, such as anodic porous alumina arrays, have the advantage to be inert *versus* animal cells and human tissues. As such, APA enables researchers to nanobiostructure a porous alumina-based matrix for developing a biomolecular (gene/protein) microarray[5,6]. Developed for the first time by Masuda and Fukuda during the year 1995, anodic porous alumina was obtained following an electrolytic route with two parallel electrodes, divided in two synthetic steps by using a 0.3 M oxalic acid solution as electrolyte[5]. APA surface is characterized by a high number of pores in the range cm^2 up to $108/cm^2$ and has a cavity with remarkable order and hexagonal pores . Several physical and chemical parameters are involved in the control of APA growth, for instance by adapting electrolyte, voltage, current density, electrode distance. APA structural variability can be indeed modulated and finely tuned obtaining pore diameters ranging from 50 to 500 nm [8]. The parametric

control of pore dimensions shows an important effect on the size modulation and due to this reason of easy reproducibility, it has suggested the possibility to confine and to spot biological fragments of complexes such as Cy3-DNA and enzymes[9]. On the base of these consolidated results previously achieved by our laboratory, it remains to further improve the funzionalization of APA structure in interacting with proteins[10]. At the moment, only few strategies can be easily followed in order to obtain funzionalized anodic porous alumina[11]. Due to amorphous state and refractive properties, APA behaves like a transparent scaffold with advantages for the following spectroscopy and interferometric steps. As such, it enables researchers to study cell-free expression as an “incubator” in conjunction to NAPPA technique[12]. Nucleic acid programmable protein array is a procedural way not linked to the cell life condition (being cell-free), which overcomes common and expensive limitations with a high purification grade, acceptable number of false positive and short time of expression. NAPPA offers brief time and real time gene expression. Proteins arrays show an easy cell-free approach, which expression time reduced to about eight hours. Antibody arrays find application to measure the abundance of targeted proteins, or as a substrate for other possible biochemical reactions[13].

Furthermore, in an APA matrix we can confine human antibody or protein[14]. For this reason, we have conjugated the anisotropic properties of APA here used not only as scaffold but also to support the superimposed fluorescence signals from spot samples to reduce constructive interference emission between two fluorescence samples, during the phase of characterization. In recent years the confinement of cDNA gene fragments of oligonucleotides in anodic porous alumina was reported[15]. Biological sample confinements open the prospective to funzionalize the APA matrix to anchor proteins with a system of three molecules. We can summarize our activity in three steps: first- step is to produce anodic porous alumina; second step of gene expression onto the APA surface is the phase of the samples characterization. by using first-, second- antibody directed to the domain region of protein expressed onto the APA surface. For gene expression, we have used the Promega kit in which is contained a TNT coupled reticulocyte Lysate System. High-density functional proteomic array was

obtained and the expression of gene (example: Jun) onto the APA surface is here presented. Common mono-use platform for an array in use is made up of nylon and glass. Slides are commonly funzionalized to anchor antibody or plasmids. Instead, the surface of glass or nylon slides for the deposition of protein is commonly functionalized by chemical or biological treatment[16]. The purpose of this research is to show an active role in the expression of plasmids on the surface of anodic porous alumina with a proven resistance[17]. Phenomena of capillarity should be avoided during the filling of pores[18]. For this reason shape and pores dimension play a major role in avoiding leaking/confinment of liquids. As such, we should spot DNA plasmids onto the APA surface together with antibodies, in order to study the expression of proteins from genes and their interaction with alumina, without altering the pore structure. Nanoarrays resulting from LB nanotemplate and piezoelectric inkjet printing of SNAP Genes utilizing E.Coli cell free expression system open the solution of problems in structural[19] and functional[13] proteomics for Cancer control.

Reagents

• Aluminium with a quadrate shape in foil 4×4 cm, was supplied from the Goodfellow Company with a purity of 99.999N.

• TNT® coupled Reticulocyte Lysate Systems was supplied from Promega. Milli-Q water with conductivity of 18 $m\Omega/cm^{-1}$ was supplied by Millipore Milli-Q system

• Teflon is used for realizing a cylindrical electro-cell with two electrodes for the synthesis of anodic porous alumina

• Platinum foil with high purity 99.95 % (Goodfellow) code n 831-722-69

• Oxalic acid $H_2C_2O_4$, assay 99% was supplied from (Sigma Aldrich) code n 241172-50G

• Ethylene glycol (Sigma Aldrich) code n 81240-1KG

• Ethanol 99,9% pure grade dried with a sodium wire (Sigma Aldrich)

• Perchloric acid 70 % (Sigma Aldrich) pure 99,999 code n 311421

• Resin to cover tips of platinum and aluminium in their rectangular shape

• Cy3 (supplied from Sigma Aldrich, Amersham Code RPN5661)

• Mouse anti-FLAG M2 antibody from (Sigma Aldrich), 1 mg/mL, clone code F1804-50UG

• TNT® R T7 coupled reticulocyte lysate system by Promega Corporation

Equipment

• -70 °C freezer (operating range -60 to -86 °C) (New Brunswick)

• 2-8 °C refrigerator (Future, UK)

• Magnetic stirrer (IKA C-MAG HS7)

• CCD camera microscope coupled with a mercury lamp, Carl Zeiss Company

• Microscope Carl Zeiss with 100x, 50x 20x, 10x.

• Incubator box (made in our laboratory). Electronic system is used to control the temperature.

Refrigerator LAUDA® LCK 4920, Model ECO RE 1225 S

Thermometer (FLUKE), Humidity meter (FLUKE 971, USA)

Pipette Gilson. Capacity of 100 µL. supply from Gilson Company

• pH/Conductivity tester HI98129 (Hanna Instrument)

• Atom Force Microscopy (made in our laboratory Nanoworld institute, Genoa, Italy)

• ImageJ free software available from web site: http://imagej.nih.gov/ij/

• Axio-Vision software 3.0 supplied from (Carl Zeiss company)

• Merck-Millipore Milli-Q system

• SNC 18 tip supply from web site store www.novascan.com

Procedure

Cut aluminum foil 5N in order to obtain a final and rectangular shape dimension of 1.5 cm x 1 cm.
Cut the template with a knife by using careful attention in order to avoid curvature and mechanical stress effects on the aluminum surface. The two electrodes should have same dimension and shape.
Rinse aluminum foil with acetone to degrease the surface.
Perform an annealing of aluminum in a vacuum stove at 420°C for 3 hours to increase aluminium ductility and template was immersing in an electrolytic bath.
Activate aluminum before treatment to remove an inert layer oxide on the surface and in more detail clean with a solution of perchloric acid 7% (V/V) with a second-electrode of aluminum in a controlled thermostatic bath by using a mixture of ace and salts.
Perform the first step of anodization using a voltage of 40 V for 1 hour.
Connect aluminum and platinum sheets to each electrode with inert clips.
Immerse both in a oxalic acid solution 0.3 M.
Add ethylene glycol to increase the conductibility of solution in quantity of 2% (V/V) referred to a final volume of 100 mL.
Agitate with a magnetic stirrer at 1000 rpm/min to disperse heating around the electrode. The current

density of solution changes its value during electrolytic processes.
Sonicate the layer of aluminium foil at the end of the first step for 5 min in a bath of ethanol to remove traces of oxalic acid.
Also during the second and final step traces of oxalic acid remain incorporated within the pores wall.
Remove disorganized layer of anodic porous alumina by etching with a solution of phosphoric/chromic acid.
Perform the second step of anodization at 40 V for 5 hours with the electrode inter distance fixed at 1 cm.
At the end of the second step, rinse alumina with Milli-Q water and with a lower clean airflow.
Put the layer thus obtained was putin a stove under vacuum with humidity control over night.
Spot on the electrolyzed aluminum surface by using micro-contact mode technique with a pen tips by a robot.
Store the sample in a refrigerator system.
Deposit a plasmid gel on the surface by an automatized system by micro-contact . On the surface of anodic porous alumina spotted with a gel plasmids was following the sequence describe in the Invitrogen kit and is here summarized.

Timing

Anodic porous alumina preparation

Step1. Annealing of aluminium foil in a vacuum stove for 3h at 420°C.

Step 2. Anodic porous alumina was prepared by electrochemical route. This is divided in two steps,

first-: one hour, seconds-: 5 h by electrochemical route

Gene expression by using TNT® rabbit reticulocyte lysate

Step 3. 8 hours

• Add DNA template and perform transcription/translation using TNT Lysate (1.5 hours at 30°C)

• Separate translation products by SDS-PAGE. (1.5 hours)

• Fix and dry gel. Fluorography optional (2 hours)

• Auto radiography (3 hours at -70°C), option phase

TNT ® rabbit reticulocyte lysate is supplied in 200 µl aliquots. Each system contains sufficient reagent

to performed approximately 40-50 µl translation reaction. Includes:

• 1ml TNT® Rabbit Reticulocyte Lysate

• 90µl TNT® Reaction Buffer • 60µl TNT® T3, T7 or SP6 RNA Polymerase (2 × 30µl in dual systems)

• 5µg Luciferase T3, T7 or SP6 Control DNA, 0.5mg/ml

• 50µl Amino Acid Mixture, Minus Methionine, 1mM

• 50µl Amino Acid Mixture, Minus Leucine, 1mM

• 50µl Amino Acid Mixture, Minus Cysteine, 1mM

• 250µl Luciferase Assay Reagent

• 1 Luciferase Assay Wells (set of 3)

• 100µl Magnesium Acetate*

*Magnesium Acetate is supplied only with Cat.# L4600 and L4601.

Promega kit was conserve at-70°C. Product is sensitive to C02 and multiple freeze-thaw cycles which may have an adverse effect on activity/performance. LAR is stable for at last 12 months if stored and handled properly

Step 3. First part of the expression with rabbit reticulocyte expression of gene (example: Jun)

Step 4. Second part of the expression protocol was the characterization of protein expression by using first–, second-

antibody anchored to Cy3 fluorochrome.

Troubleshooting

The pore funzionalized on a matrix of anodic porous alumina, before to deposit plasmids on the surface, prevent the loss of gene expression material from the surface.

Anticipated Results

The results obtained (Figs. 1-6) report primarily the matrix characterization of anodic porous alumina obtained from an electrolytic process with topographical images of the surface, during the phase of validation with atomic force microscopy in contact mode by using a SNC18 tip, which are in according with the results introduced by CCD camera images coupled with microscopy. An APA image reported of the sample layers was acquired by using CCD camera before the robot printing procedure with and afterward were put in comparison with Fig.1 b) were is clearly visible a circular shape on the surface. We consider that this grey circular shape zone, depend from an altered refractivity behaviour of the light on the APA surface without treatment and with sample spots. Images from only APA and with a circular spot are reported , don’t show altered or damage effects on the surface. The fluorescence signals acquired from the slide samples depends from the concentration of Cy3 fruorophore in solution, formation of dimers and trimmers, proteins conformation on the APA surface .17 In

particular, if the protein has a good degree of hydration and an optimal conformation in this environment, a large number of domains are exposed to primary antibody, second, and to Cy3. This can have an influence on the fluorescence signal yields. Furthermore, we know from our experiments that the fluorescence signals from APA surface treated with Cy3 must be attributed to the complex linkage protein-antibody 1-antibody 2 and with Cy3 and not from native fluorescence of APA or from fluorophore deposited on the surface of APA.18 Namely, the Cy3 fluorochrome unlinked to the complex was completely washed out from both holes and pore walls, give no fluorescence after three forced subsequent rinsing steps with Milli-Q water. The effects of a phosphate buffer, which were received in the expression kit and were preliminarily tested on APA, were as follows: the CCD images showed that the APA architecture order is not affected by this treatment. After the treatment with a solution of fluorochrome Cy3, Axio Vision 3.0 software in live modality was used to acquire fluorescence images after a frame step of 40 ms, the characterization phase was monitoring for 6 min. A modality of signals attenuation is activated but fluorophore concentration is included in a range to avoid effect of saturation. Circular spots with irregular shapes were detected on the APA surface and are reported in Fig. 3 a). A magnification of a single spot is reported in Fig. 3 b). Traces of robot pens extremity are clearly shown in the concentric distribution (in particular in the right spot) and by the radii crossing the spots; correspondingly, the fluorescence signal of the protein expression is lower in these regions. The fluorescence signals intensity is different into the domino of the circular spot.

We addressed these behaviours to free arrangement of proteins in the growing process and their 3D folding, which influenced the full fill of pore structure as well as the geometry of the genes deposition by the robot. Furthermore, the spot enlarged c observed close to the left spot. Conventional NAPPA on flat surfaces showed neither these side structures nor the fingerprint of the robot pen tip. At present, these findings in material as porous as APA point out the potential effects in the hydro-dynamical conditions between the solution drop from the robot pen and the hydrophobic nature of the APA surface.19 In a flat surface, the solution deposited by the pen tip attains homogeneous distribution before water evaporation, cancelling the tip shape memory. In APA, the diffusion trend is prevented

by pores, which confine the solution content within pore. The fluorescence is present only in the zone of circular spot. This confirmed that the cy3 treatment interest only circular spot and is complete removed after rising of slides.

In the application of bacterial PURExpress (Figure 7) to NAPPA a template double-stranded DNA containing the gene of interest is fused to a SNAP tag and to an upstream T7 promoter when immobilized in APA pores. By adding the PURExpress reconstituted cell-free translation system, the template DNA will be transcribed into mRNA, and then translated into a fusion protein containing the N-terminal SNAP tag and the C-terminal target protein. In the same APA pore, the SNAP tag allows the synthesized protein to bind to its own template DNA via the BG linkage, thus immobilized the target protein. The immobilized target protein is allowed to interact with a mixture of query proteins and after the binding reaction, the unbound proteins are washed away and the target protein complex is released by cleaving the template DNA. For nanocrystallography, adding a small amount of the DNA endunuclease will cleave the template DNA and release the fusion protein. Alternatively, a site-specific protease can also be used to cleave between the SNAP tag and target protein, releasing the untagged target protein to form nanocrystal by in situ interaction.

APA SNAP microarray coupled with LB nanotemplate and cell free expression system (Figure 8)[19,20] is being used as a platform for structural and functional proteomics, where proteins are being expressed by the Pure-Express cell-free system produced by New England Biolab.

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Associated patents.

Bragazzi NL, Nicolini C, Peshkova E. APA-SNAP nanoarrays per proteomica LB-basata (strutturale e funzionale) e per vaccinologia. ITGE20120080 (A1) — 2014-02-10 (http://worldwide.espacenet.com/publicationDetails/biblio?FT=D&date=20140210&DB=worldwide.espacenet.com&locale=en_EP&CC=IT&NR=GE20120080A1&KC=A1&ND=5).

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Associated book chapters

Nicolini C, LaBaer J. Nanotechnology applications of Nucleic Acid Programmable Protein Arrays, in Functional Proteomics and Nanotechnology-based Microarrays, Vol. 2 (Eds. Nicolini C., LaBaer J.) Pan Stanford Series on Nanobiotechnology, London - New York - Singapore, 2010, Ch. 1, Thomson ISI Web of Science

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Figures

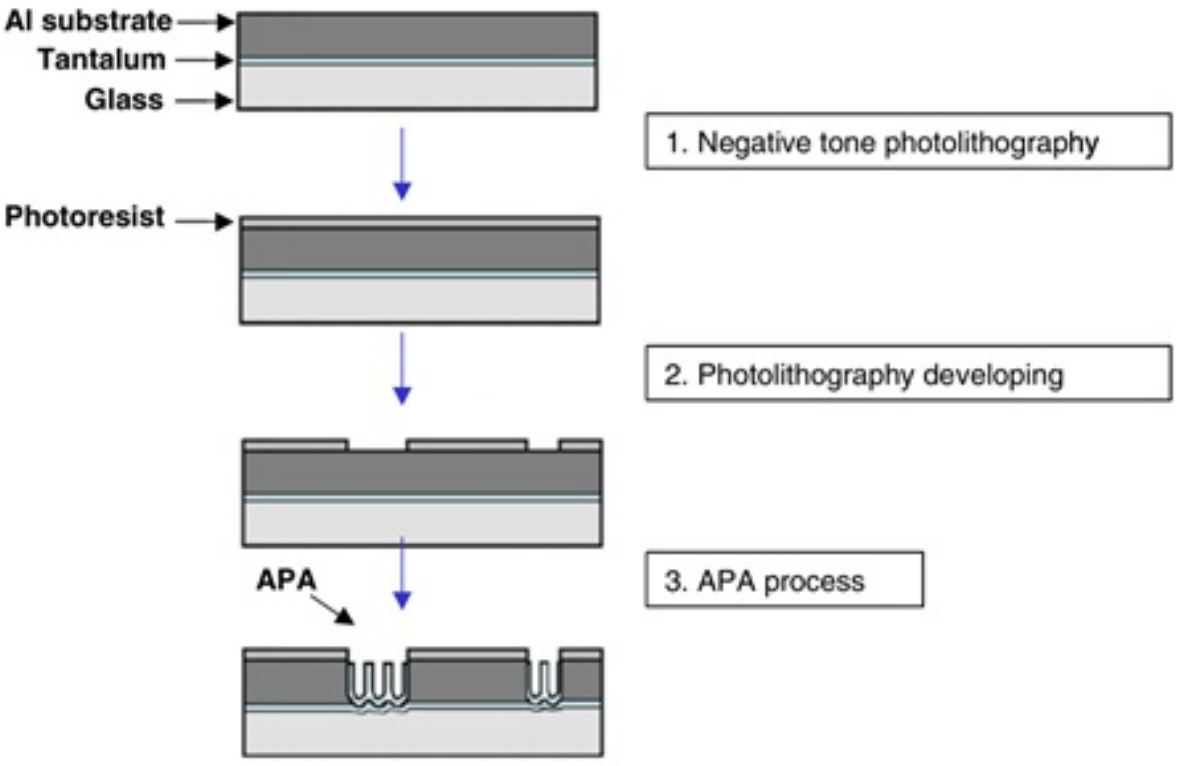

Figure 1

APA microstructuring process Schematic diagram of APA microstructuring process (from Grasso et al., 2006).

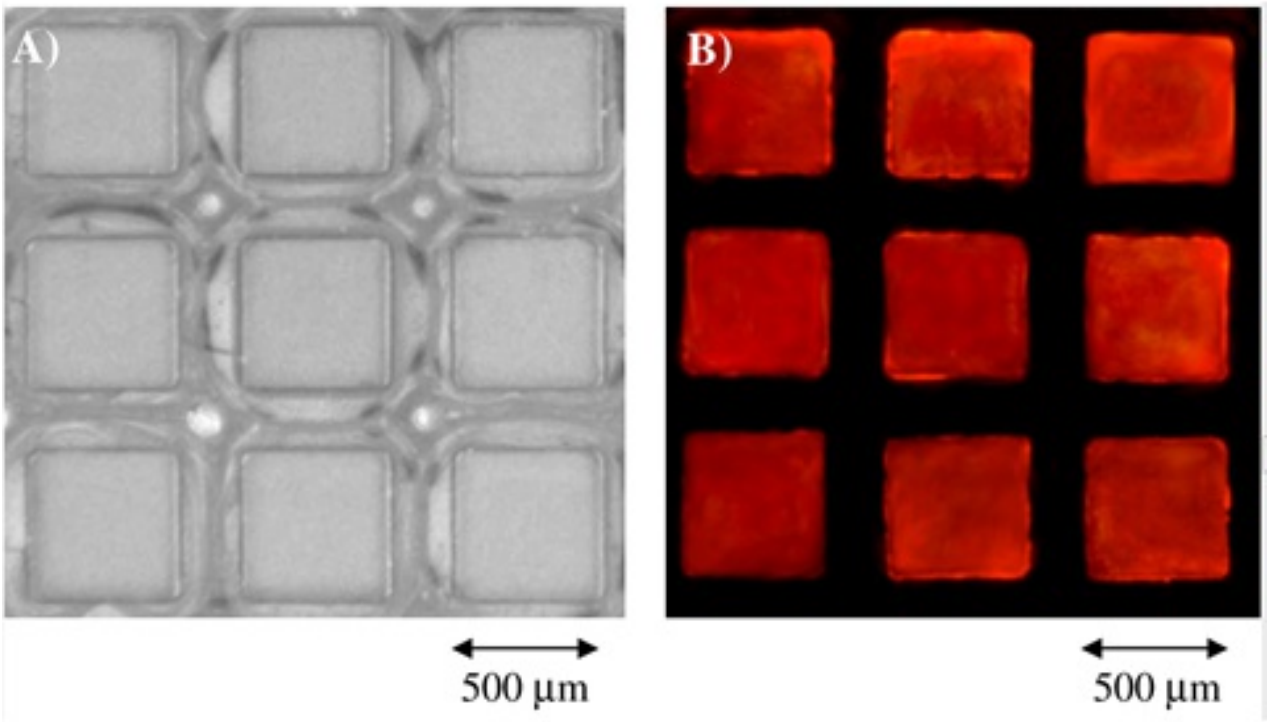

Figure 2

APA matrix APA matrix before (a) and after interaction with Cy3 labelled cDNA (b). The detail of nine spots (fluorescence microscope image, magnification 2×, filter set no 15) shows that the fluorescence appears on the APA only when DNA phosphates and PLL are present and is lacking in the control solution containing PLL without DNA (from Grasso et al., 2006).

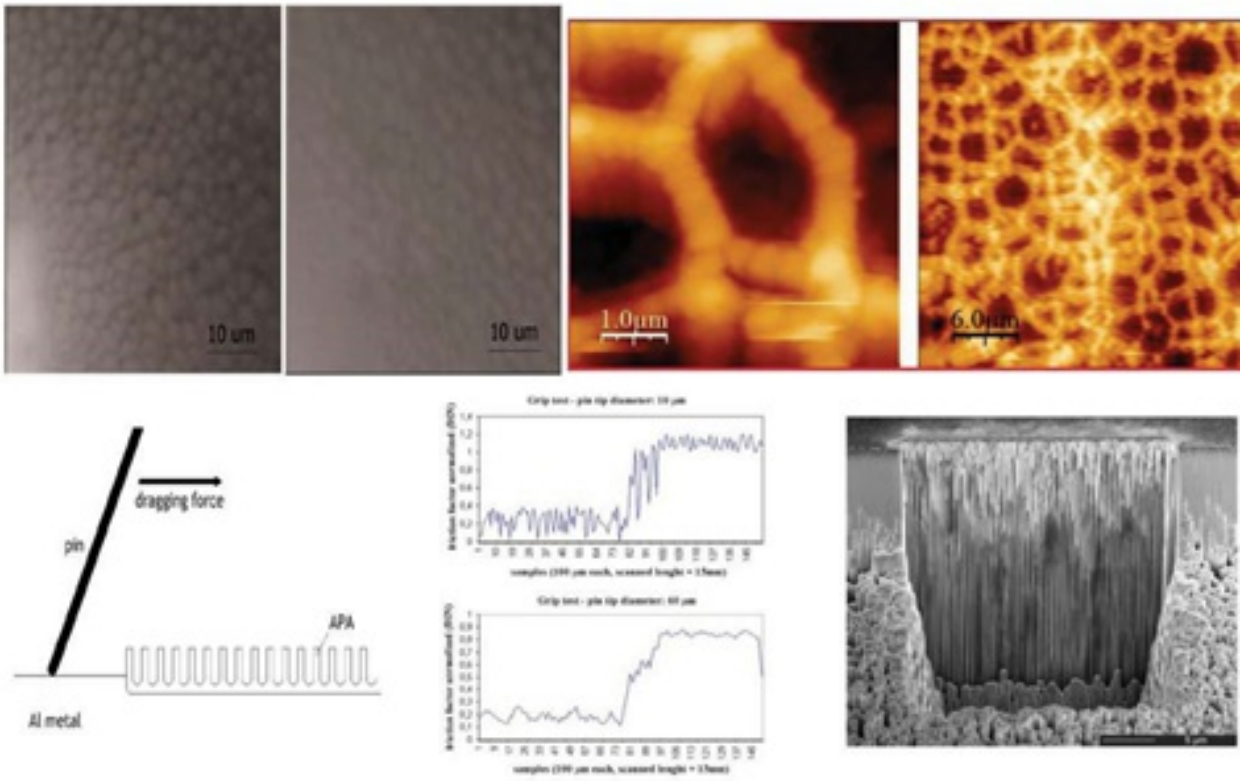

Figure 3

APA patterns (left, above) Horizontal cross-section, via AFM following fluorescent dye removal by forced rinsing; (right, above) APA spots formation at two different magnification, in presence of aluminum purity 99.999%, acid concentration 0.5 M, reaction time 30', voltage 46 V and distance between electrodes of 1 cm; (left, below) setup for grip test carried out to characterize mechanical properties of APA; (right, below) result charts for grip test with different tip sizes. The same test has been carried out with two test pins, with different tip diameters , respectively 10 (above) and 60 (below) µm. The drag path length was 15 mm, and exactly in the middle of the path the surface changed from aluminum metal to APA. It's clearly visible the increase of the friction factor, particularly for the thinner tip (this should be due to the more similar size of the tip and the APA pattern) (from Nicolini et al., 2013a; Nicolini et al., 2013b).

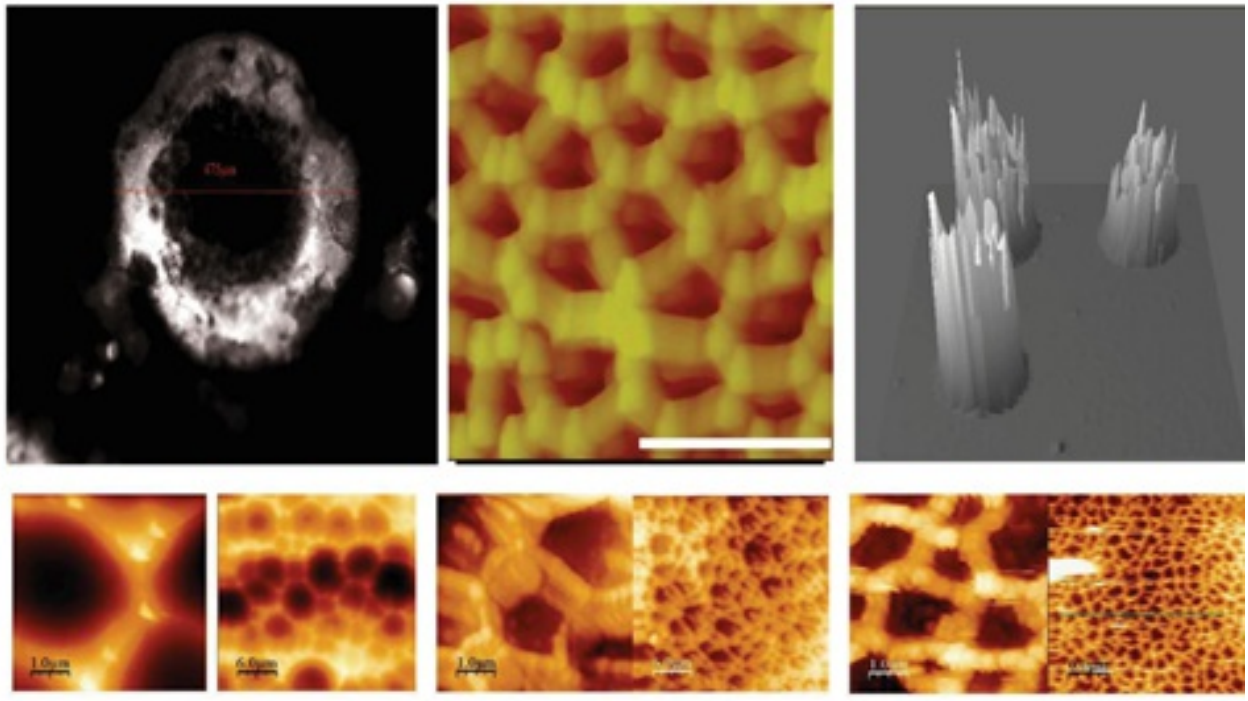

Figure 4

APA and NAPPA fluorescence (Above) Single NAPPA fluorescence gene spot printed on APA after its expression in 2D (left) and in 3D (right). For comparison Atomic Force Microscopy of APA cross-section on glass in 2D is shown in the center. (Below, from left to right) To vary pore size and depth using Aluminum purity 99.999%, we vary from left to right the acid concentration (0.5 M, 1M,1M) , the reaction time (150', 30', 120'), the voltage (30 V, 30V, 40V), the distance between two electrodes (1 cm, 2cm, 1cm) (from Nicolini et al., 2013a; Nicolini et al., 2013b)

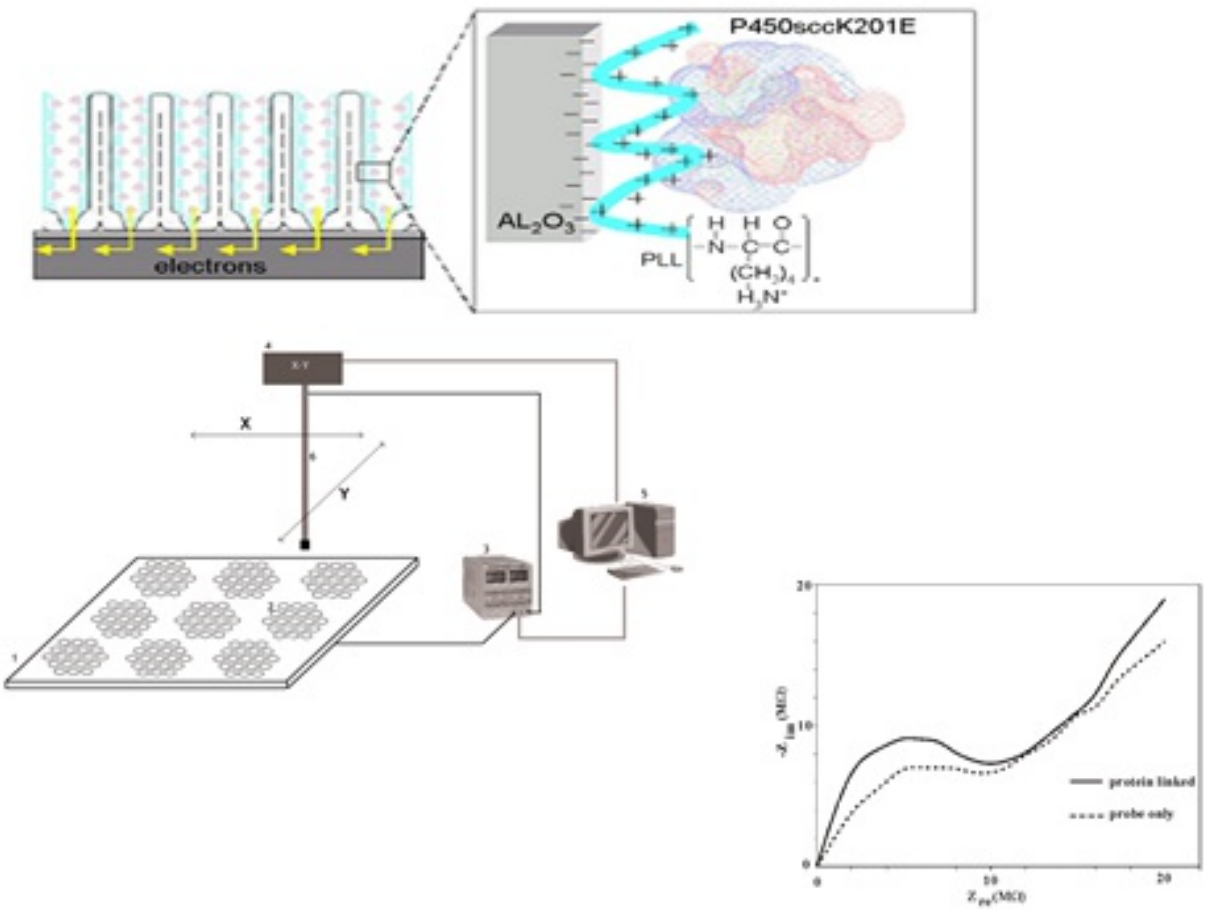

Figure 5

APA functionalization (above) APA functionalised rhodium–graphite s.p.e. working electrode. Schematic view illustrating the direct electron transfer between the cytochrome P450scc catalytic ‘core’ and the APA modified working electrode. In the box is shown the specific interaction between the cytochrome P450scc negative surface (blue) and the positive charges of poly-l-lysine (from Stura et al., 2007). (left,middle) Set up to analyze NAPPA elements using impedentiometric measurements: 1 – Aluminum substrate, serving also as counter electrode. 2 – APA spot, obtained by lithography, with biomolecules bound 3 – AC signal generator, controlled by PC. 4 – XY bidimensional actuator, controlled by PC, positioning the scanning electrode upon spots. 5 – PC, controlling bidimensional mover and AC signal generator. 6 – Scanning electrode, dipped in the solution containing NAPPA and buffer. (right,below) Impedance spectroscopy plots in two spots of APA surface, one with protein hybridized to the probe molecule and another with probe only. Frequency ranges

from 1 Hz to 100 KHz, voltage applied was 400 mVpp

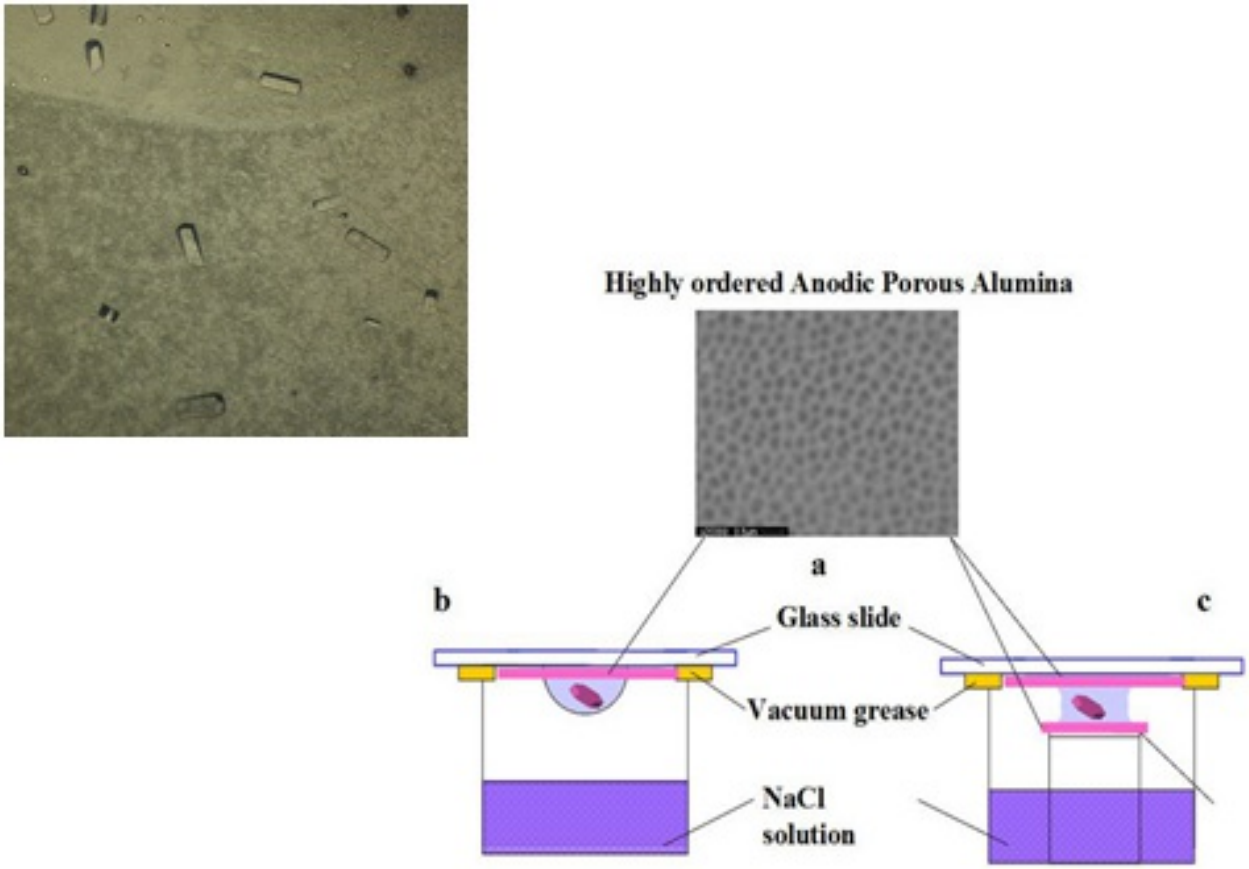

Figure 6

APA template for crystallization The APA template crystallization by hanging drop (b) and sandwich drop (c) modified techniques. Scanning electronic microscopy (SEM) picture (top view) showing highly ordered anodic porous alumina on the surface of the template, resulting at the end of photolithographic microstructuring technique and two- step anodization process (a), as described in the main text (from Pechkova et al., 2015).In the upper corner are shown the lysozyme crystals being produced by APA template.

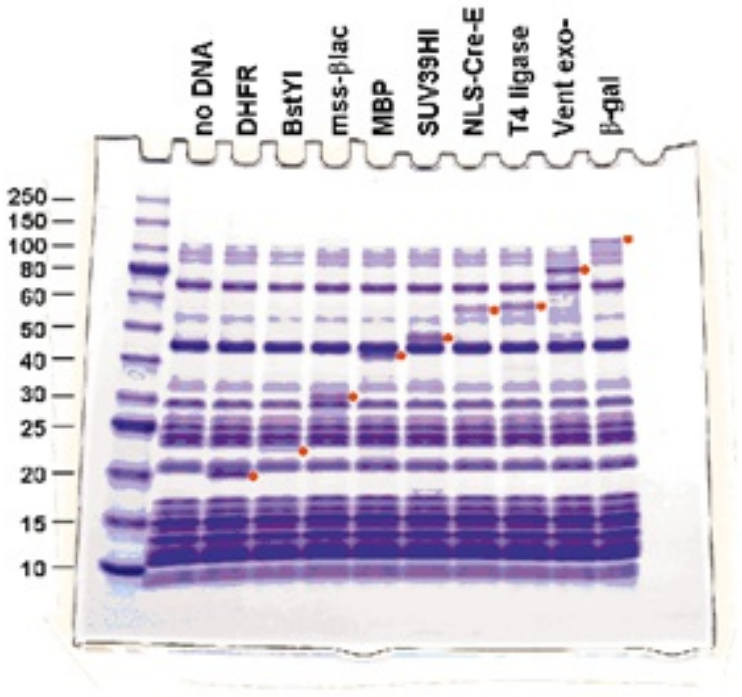

Figure 7

APA and PURExpress In vitro translation of a diverse set of target proteins using PURExpress system in 25 µl (from Pechkova et al, 2010).

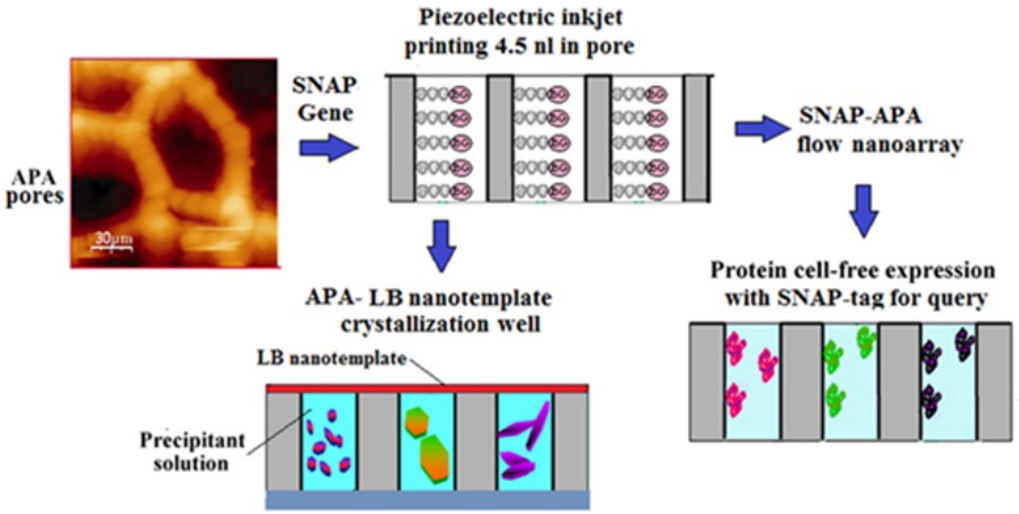

Figure 8

APA Microarrays utilizing piezoelectric inkjet and APA LB for nanocrystallography APA

Microarrays utilizing piezoelectric inkjet and APA LB for nanocrystallography. A SNAP–APA

flow application for funcional proteomics is also shown.

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Nanoprobe NAPPA Arrays for the Nanoconductimetric Analysis of Ultra-Low-Volume Protein Samples Using Piezoelectric Liquid Dispensing Technology

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