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MULTI-AUTHOR REVIEW

Nanobiochips

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Abstract The actual progress towards biological chip devices consisting of nanostructured functional entities is summarized. The practical aspects of molecular nanobiochips are discussed, including the main surface chemistry platforms, as well as conventional and unconventional fabrication tools. Several successful biological demonstrations of the first generation of nanobiochip devices (mainly, different nanoarrays) are highlighted with the aim of revealing the potential of this technology in life sciences, medicine, and related areas.

Keywords Nanobiotechnology · Biochips · Protein nanoarrays · Protein complexes · Nanolithography

Nanobiochip as a concept

A nanobiochip can be arbitrarily defined as a device consisting of integrated biological and/or biologically active artificial structures with their dimensions below typical sizes of cell organelles. Many materials display substantially different physical and chemical properties at particle/structure dimensions below 100 nm, as compared to the "bulk" material properties. Therefore, the constituent features of nanobiochips should be within that critical size range to make the technology more beneficial than most of the microarray or other bioanalytical devices available today. In terms of its applicability in biology or medicine, the nanobiochip can be envisioned as a tool designed to interact specifically with or manipulate biomacromolecular quaternary structures, cell membrane domains, and other biological architectures in a programmable, stimuli-responsive manner. Such devices can be designed to interact with single cells or their organelles for analysis and engineering purposes, or they can be used for biophysical studies of structure-function relations. In this review we will summarize the recent efforts in prototyping and fabrication of the first generation of biomolecule-based nanodevices, highlighting selected successful biological applications of "soft" nanopatterned architectures. Lab-on-a-chip platforms and micro/nanofluidic components such as nanochannels, nanopore, and nanowire arrays belong to another distinct activity area in the nanobiochip development and are presented elsewhere [1, 2].

Surface platforms

Similarly to microelectronic devices, the simplest nanobiochip has a planar "circuitry-like" design, with different physical, chemical, and biological elements arranged in a defined layout on a solid or soft (e.g., polymeric) support. The precision of the chip layout (practically a few nm in *xy* and a few Å in *z*-direction) and the sensitivity of the constituent elements to their environment (for example, the stability of proteins, nanoparticles, spectral properties of quantum dots, metallic nanostructures, etc.) set specific requirements for the quality of the immobilization platforms. The overall formation of nanoscale assemblies is governed by the intermolecular and interparticle forces [3, 4] that have to be precisely controlled in order to maintain the distinct biological function and to build a reliable device. Therefore, the control over the interfacial properties and different interactions between the nanobiochip components relies on advanced surface chemistries.

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Different chemical modification strategies for biomolecule immobilization have been recently discussed elsewhere [5–7]. Herein we will briefly present the main groups of surface platforms that are most convenient for fabrication of nanobiochips.

Self-assembled monolayers (SAMs) of bifunctional molecules on metallic and oxide substrates have been extensively studied since the 1980s, and they have become an important form of nanotechnology, including diverse biological applications [8, 9]. The major advantage of the SAMs is Angstrom precision in positioning of chemical groups at the solid/organic interface. Also, SAMs are relatively stable as compared to other surface-supported molecular platforms because they are strongly (most often covalently) pinned to the substrate via a reactive head group, which typically is thiol, disulfide, or silane. Therefore, SAMs are compatible with the majority of standard and emerging fabrication techniques (see below, section Fabrication tools). So-called mixed SAMs can be obtained via surface adsorption of a mixture of two or more bifunctional compounds with different terminal entities, an approach that allows combining chemically and biologically inert molecular modules [such as polyethylene glycol (PEG)] with different terminal functional groups (carboxyl, amine, maleimide, azide, etc.) or biospecific tags, ligands, ssDNA, enzymatic co-factors, etc. Docking of biomolecules at such a biocompatible interface can be precisely controlled in terms of their orientation, surface density, conformation, and functional activity. In mixed SAMs, nanoscale phase segregation can also be induced by synthetically inbuilt intermolecular interactions to obtain specific domains for templating of different molecules [10].

Although less structurally defined, polymeric grafts and coatings also are commonly used in nanobiochip development. Modifications of solid chip supports by polymers may be advantageous, as often practical problems arise related to the efficiency and reproducibility of silane SAMs on standard surfaces such as glass [11]. In particular, long or short poly(ethylene glycol) [12] or its copolymers [13] provide a reliable biocompatible matrix for minimizing nonspecific binding. Hydrogel layers can be synthesized and structured on surfaces by a variety of fabrication techniques [14]. Hydrogel gradients [15] and nanoarrays [16] are suitable for controlling the surface density of immobilized proteins on a nanobiochip.

Supported lipid monolayers, bilayers and more complex membrane-like assemblies provide a dynamic surface platform that is attractive for mimicking the natural environment for transient biomolecular nanostructures and sophisticated cellular transport and signaling machineries [17]. Supported lipid membranes can be formed either on flat or micro/nanofabricated solid surfaces, on different polymer cushions, SAMs, protein layers, etc. [18–20]. Different lipids can be introduced into a supported membrane (e.g., for obtaining its asymmetry, domain formation, etc.) by vesicle fusion, self-assembly, Langmuir-Blodgett deposition, and other techniques. Vesicle micromanipulation allows generating complex nanofluidic networks based on lipid membranes [21, 22].

The above-listed surface platforms allow advanced surface engineering via further functional modifications, enabling more complex supramolecular or biomimetic approaches in nanobiochip design. They extend the capabilities of regular chemical immobilization approaches as they allow docking and multiplexing of biomolecular entities on the nanobiochip with controlled orientation, high specificity, and tunable affinity. The power of such new functional surface platforms can be illustrated by so-called multivalent surfaces. For example, Reinhoudt and coworkers described SAMs consisting of β-cyclodextrin [23], a molecule that is capable of creating host-guest interactions with a variety of small, organic entities through hydrophobic interactions. Owing to this property, the β-cyclodextrin SAM has been employed as a chemical printboard for micro- and nanopatterning of functional entities via multivalent interactions [24, 25]. Multivalent chelator molecules, developed by Tampe and Piehler, are another related concept that is in particular important for stable and oriented attachment and manipulation of native recombinant proteins [26]. This chemical tool has been further developed for light-triggered, multiplexed patterning of proteins under native conditions [27, 28].

Fabrication tools

Conventional fabrication methods

The dominating technological processes in micro/nanodevice fabrication are photolithography and electron beam lithography (EBL). Although foreseen for development and mass production of semiconductor devices, these techniques have been successfully employed in combination with the above-mentioned surface platforms for prototyping of nanobiochip architectures. Since organic layers are radiation-sensitive, biospecific nanopatterns can be fabricated via selective decomposition/activation and a subsequent chemical modification for recruitment of proteins and other biomolecules. For example, Zharnikov, Grunze, and coworkers studied in detail electron-induced changes in SAMs [29, 30] and developed a method called electron beam chemical lithography [31]. Their approach has been applied for in situ synthesis of multivalent chelator arrays and assembly of His-tagged proteins [32]. The electron beam can be employed for local functional inactivation of proteins, as demonstrated by EBL patterning of

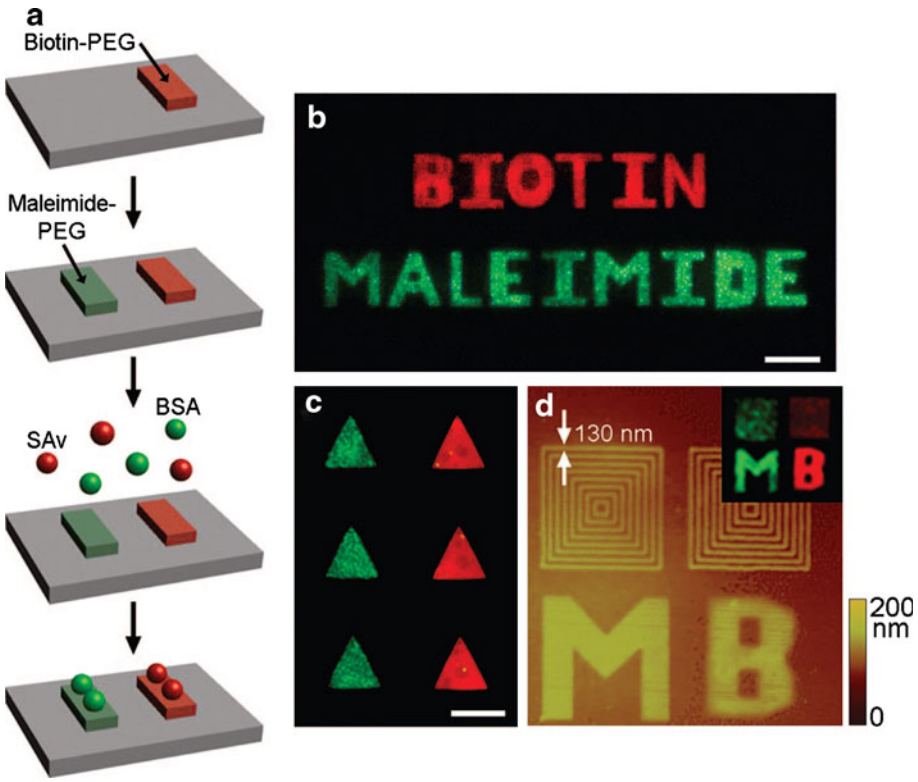

Fig. 1 Electron beam nanolithography (EBL) can be used for high precision fabrication of chips containing different chemistries for selective immobilization of several different proteins. Example of dual-protein patterning by EBL. **a** Biotin-PEG and maleimide-PEG are cross-linked next to each other. BSA, which contains one free cysteine, conjugates to the maleimide-PEG, and streptavidin (SAv), which binds to the biotin-PEG, are attached simultaneously from the same solution. BSA is visualized using the appropriate antibodies. **b**, **c** Fluorescence overlays demonstrating attachment of the two proteins to microscale patterns (*scale bar* = 10 μm). **d** Nanoscale patterns of the two polymers are visible in the height image taken with an atomic force microscope in tapping mode. Concentric squares of maleimide-PEG and biotin-PEG have line widths of 130 nm. Letters "*M*" and "*B*" are written below the patterns to denote the respective functionalized PEGs. The *inset* displays a fluorescence overlay of the nanoscale patterns with attached BSA and SAv. Reproduced from Christman et al. [34]

fibronectin on silicon substrates [33]. Further on, electron beam-induced cross-linking of functional PEG derivatives to silicon surfaces is an interesting strategy for precise chemical nanopatterning [34]. Multicomponent protein nanopatterns on the same chip have been produced by this technique, achieving the smallest line width close to 100 nm (Fig. 1).

Interestingly, photolithography can be performed in aqueous environments yielding patterns of supported lipid bilayers [35, 36]. Although resolution of such a process is limited by the light diffraction, rather complex multifunctional lipid microdomains can be generated as a pre-structured, membrane-protein-friendly surface for further assembly of nanoscopic bioarchitectures. Photolithography and EBL can also be combined with different nanoparticle self-assembly phenomena. For example, Spatz et al. developed block copolymer micelle deposition on different types of surfaces [37]. This so-called block copolymer micelle nanolithography is in particular advantageous for functionalized inorganic nanoparticle array formation when the desired feature size is in the range from 1 to 12 nm.

Unconventional fabrication tools

Standard fabrication and patterning processes require environments, chemicals, and temperatures that most often are not compatible with the very low stability of most of the biological components. Also, there are practical difficulties that complicate manipulation of patterned biomolecules in their native state. Other drawbacks of EBL and photolithography are the high equipment and facility costs, especially in the prototyping phase. These and other factors create a divide between the state-of-the-art in nanofabrication techniques and the potential of nanotechnologies in life sciences. Therefore, since the mid-1990s a wave has been seen in the development of new methods and tools [38], often referred to as unconventional, that are more suitable and flexible for fabrication of functional bioarchitectures. The driving idea behind these technologies

is to create compact and cost-effective biochip fabrication devices and tools more compatible with biomolecules and cells.

Soft lithography

Microcontact printing (μCP) is a typical example of the family of direct patterning methods generally known as soft lithography [39]. Direct transfer of SAM-forming molecular "inks" with elastomeric relief stamps was first introduced as a simple and reliable substitute for replication of chemical and topographic patterns beyond microelectronics laboratories. Microcontact printing is not limited to synthetic compounds, but it also enables contact transfer of patterns consisting of different biomolecules. Optimization of the mechanic properties of the stamping tool and synthetic ink design enables fabrication of surface features close to 100 nm [40–43]. An important advantage of this technique is the capability to pattern relatively large surface areas ($\sim$cm^2), and wafer-scale production in principle is possible. The ultimate resolution of the μCP process was demonstrated by Delamarche and co-workers: single antibodies and green fluorescent protein molecules were arrayed on a glass surface using stamps with protruding spherical features [44]. Similar printing of proteins has been employed in templating of single lipid vesicle arrays for functional analysis of membrane proteins [45]. Elastomer-based lithography techniques can be further enhanced by combining them with capillarity and colloidal self-assembly to produce functionalized nanoparticle architectures with line widths of close to 10 nm [46, 47].

Scanning probe lithographies

Direct positioning, machining, and manipulation of biomolecular entities at sub-100 nm scale is obtained by precision patterning tools such as scanning probes used in atomic force microscopy (AFM). A family of scanning probe lithography (SPL)-based methods is emerging as a powerful platform in nanobiotechnology. For example, nanografting is suitable for fabrication of functional nanostructures via first grafting of thiol molecules with a specific chemical group within a matrix SAM on gold [48], followed by covalent or noncovalent attachment of antibodies or other proteins [49, 50]. Similarly, recombinant proteins are grafted directly within a "matrix" protein layer attached to a multivalent surface, and the complete fabrication process can be implemented in buffer, i.e., native conformation of proteins is maintained (Fig. 2) [51].

Dip-pen nanolithography (DPN) is an SPL technique for direct writing with an AFM tip coated with molecular "inks" on a variety of chemically reactive or non-reactive substrates [52, 53]. It allows nanopatterning of charged or chemically reactive compounds for subsequent adsorption/covalent linking of proteins [54, 55]. Membrane proteins such as photosynthetic complexes can adsorb from crude extracts preferentially to amine-terminated SAMs printed by DPN [56]. Individual virus particles can be immobilized on charged DPN-made nanopatterns via coordination chemistry [57], or they can be docked to the DPN-made patterns covalently [58]. Alternatively, it is possible to directly write patterns of biomolecules such as DNA [59] or lipids [60]. Robust proteins (e.g., lysozyme or immunoglobulin) can be printed directly with a scanning probe via covalent or electrostatic interactions with the substrate [61, 62]. Positive patterning of large extracellular matrix (ECM) molecules such as collagen and collagen-like peptide has also been demonstrated, achieving 30–50-nm line widths [63]. Proteins tagged with several histidine residues (His-tagged proteins) can be printed on nickel oxide surfaces [64]. The latest development of DPN instrumentation opens for highly parallel molecular fabrication using millions of pens simultaneously [55]. The concept of DPN was further transferred into polymer pen lithography (PPL) [65]. This technique is based on using polymer pen arrays rather than solid SPM tips, and the throughput of the fabrication process was shown to be 150,000 features per second for sub-100-nm structures. Such polymer pen pyramid arrays ($\sim$11 million) can be used for direct writing of molecular nanostructures on an entire silicon wafer surface. These technological innovations are important for development of nanobiochips for cell culture experiments in which sample throughput and cost are important limiting factors for more broad biological applications.

A more recent addition to the SPM-based chemical nanopatterning methods is thermochemical nanolithography. The concept is based on a resistively heated AFM cantilever that induces well-defined chemical reactions in a thin polymer films [66]. Thermochemical nanolithography can write and read in situ chemical nanostructures at speeds faster than 1 mm/s, with sub-15-nm patterning resolution. More recently, it was applied for multiprotein patterning on glass surfaces [67]. The technique overcomes limitations of the more common SPL approaches such as DPN, as it is not sensitive to uncontrolled ink diffusion and irregular diffusion due to tip coating imperfections.

Other examples of emerging techniques relevant to biomolecular nanoscale patterning and nanobiochip fabrication include electrohydrodynamic jet printing, which is suitable for developing complex patterns of robust biomolecules like DNA, achieving 100-nm resolution [68]. The laser scanning lithography-mediated desorption technique proved powerful in generating submicrometer-sized cell adhesion ligand features [69]. Nanoimprint lithography (NIL), which otherwise is being developed as an alternative tool in nanoelectronics and material science, has been

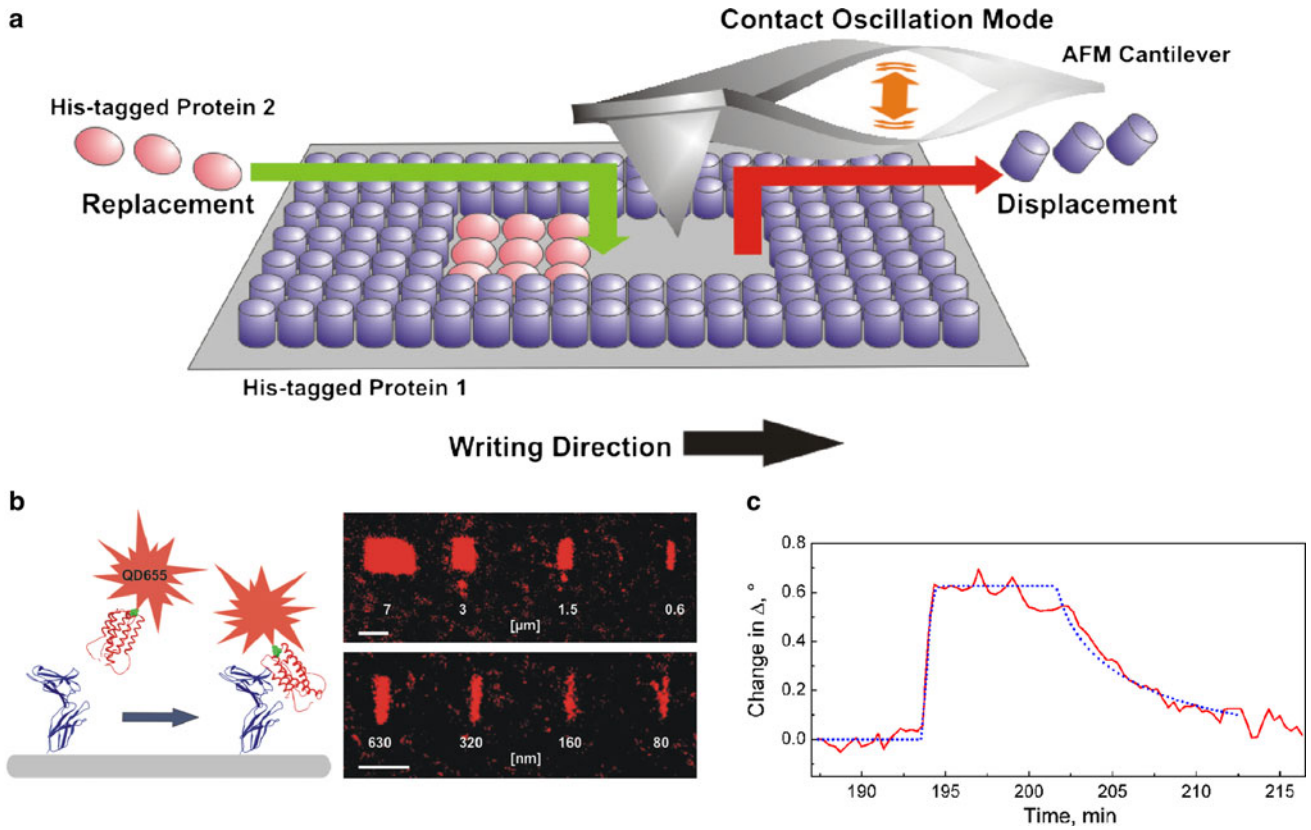

Fig. 2 Toward "custom-made" protein assemblies and dissection of multiple protein interactions. **a** Histidine-tagged proteins can be precisely organized under native conditions by tools such as scanning probe nanolithography and multivalent chelator self-assembled monolayers. b Fluorescence microscopy detection of the specific interaction between nano-arrayed human type I interferon receptor (ifnar2-His_{10}) and interferon-α2 labelled with quantum dots (reproduced from Tinazli et al. [51]). **c** Commercial equipment also allows probing protein-protein interactions in similar nanopatterns quantitatively, in a non-labeled manner, an analytical approach that will further expand with the advances in the area of nanoplasmonic biosensing [93]. For example, imaging surface plasmon resonance was used for real-time characterization (red curve) of ifnar2-His_{10} and interferon-α2 interaction within a ROI of 16 × 16 μm^2, patterned by dip-pen nanolithography. The kinetics was fitted (*black curve*) by a receptor-ligand interaction model, which yielded an association rate constant k_a of $\sim 2 \times 10^5$ $M^{-1}s^{-1}$ and a dissociation rate constant k_d of $\sim 1 \times 10^{-2}$ s^{-1} (reproduced from Rakickas et al. [95])

successfully applied for fabricating patterns of a biotinylated polymer [70] or the NTA chelator [71]. More recently, the use of NIL tools has been demonstrated in developing silk-based biomaterials with inbuilt photonic structures [72], a platform potentially useful for flexible substrate based nanobiodevices.

Biomimetic self-assembly in the toolbox

Although the performance of the fabrication processes in terms of resolution, registry, and multiplexing, in principle, already meet the requirements for construction of architectures consisting of multiple biomolecular entities, rational design and fabrication of artificial protein complexes on a chip remain a challenge. Inherent flexibility of the biological soft matter requires more practical options in the fabrication toolbox that are based on molecular self-assembly, molecular recognition, and scaffolding rather than on direct positioning and chemical attachment.

One powerful approach that in the future may provide such presently missing fabrication means is the emerging area of DNA nanotechnology. This self-assembly approach allows obtaining well-defined artificial nucleic acid architectures dubbed "DNA origami" [73–75]. Specific oligonucleotide strands can be obtained via computational and synthetic design, and upon base pairing they can form complex 2D and 3D structures and even functional nanodevices [76]. Chemically active groups, biological tags or tag-specific groups can be incorporated into these DNA architectures for scaffold-like assembly of other molecular components, for example, multiprotein complexes. DNA origami is in particular suitable for precision assembly of susceptible biomolecular components at resolutions close to 10 nm. In the first attempts to implement these concepts, DNA nanogrids with biotinylated nods already allowed generating arrays of single streptavidin molecules with a spacing around 20 nm [77]. Single molecule-level chemical reactions were successfully performed and imaged on

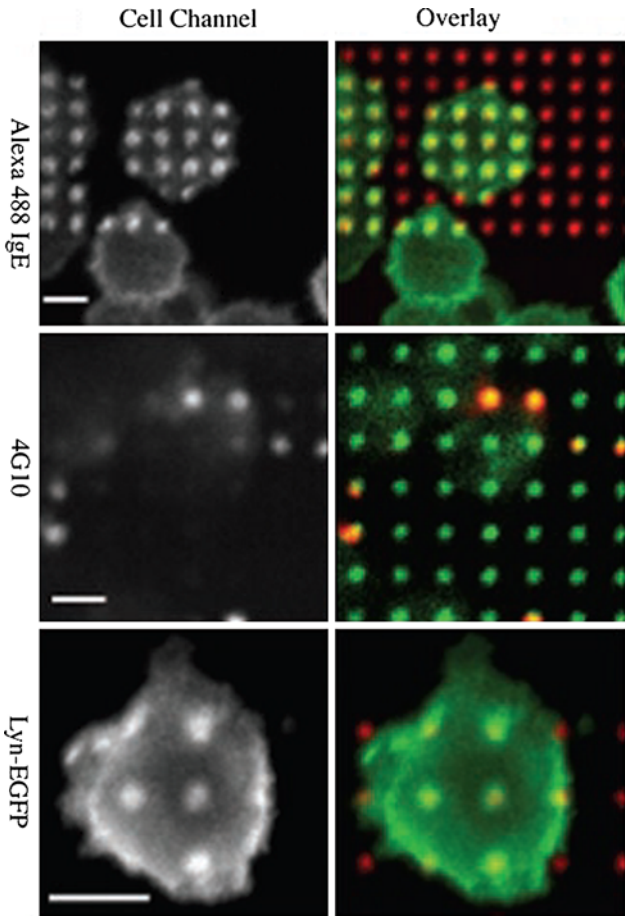

Fig. 3 Monitoring of redistribution of cellular components with IgE receptor complexes in RBL cells adhered to a micropatterned lipid bilayer chip. Confocal microscopy images of RBL cells stimulated by lipid patterns with 10 mol% 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-[6-[(2,4-dinitrophenyl)amino] hexanoyl]. Localized IgE, 4G10 indicating tyrosine phosphorylation, and kinase Lyn-EGFP related to the lipid pattern. Sub-cellular arrays of active substances are instrumental in revealing cell membrane structural organization and lipid raft dynamics (reproduced from reference [83])

such a solid-supported DNA grid [78]. It has been shown that the DNA origami, in turn, can be precisely positioned on solid templates fabricated by standard semiconductor processes, and the orientation of individual DNA structures in such experiments was performed with an angular dispersion of ±10° [79].

Examples of applications in biology

In the following chapter we will highlight a few examples of the first generation of different nanobiochip devices with the aim to illustrate the state of the art and also the practical implications for certain areas of life sciences. In particular, single-cell research-oriented applications of nanobiochip platform have been a vivid area of activities so far. For example, Spatz et al. have established a nanopatterned cell adhesion substrate architecture that is based on generating of gold dot arrays containing RGD peptides. This architecture has been used for modulated clustering of the associated integrins [80], as the nanodot deposition and patterning technology allows to change precisely the spacing between the individual clusters in the scale from about 30 to 80 nm [81, 82]. Soft surface structures for direct interfacing with living cells and controlling of receptor-mediated signaling were reported by Baird, Craighead, and co-workers [83]. They targeted the problem of cell membrane domain formation via antigen-mediated crosslinking of immunoglobulin E (IgE) receptor complexes by employing surfaces that contained micrometer-sized patterns of lipids. The chips allowed direct monitoring of redistribution of cellular components with IgE receptor complexes that were concentrated at these patterned bilayers (Fig. 3). In a similar approach, biomimetic membrane patterns obtained by DPN printing of chelator or biotin-terminated lipids have been used as substrates for selective adhesion and activation of T-cells [84]. The use of tools such as DPN allows shrinking the active feature sizes on the nanochip surface close to the actual dimensions of cell membrane domains such as lipid rafts (sub-200 nm) (Fig. 4).

Subtractive printing, which is a version of soft lithography, was developed by Delamarche et al. and allows large-scale nanopatterning of proteins [85]. Using this technique, single filamentous M13 bacteriophage virus particles could be immobilized and aligned over cm-sized surface areas by employing nanoarrays of antibodies [86]. Although the cited work did not include a relevant biological application, the potential of this type of virus nanopattern in single-cell level virology has been demonstrated by another study that reported on similar structures made by DPN. Namely, nanoarrays of antibody were printed and used for recognition-directed assembly of recombinant parainfluenza virus SV5 [87]. The virus-loaded chip could be used for microscopy analysis of pathogenic infectivity at the single-cell, single-particle level [88].

Nanobiochip architectures have shown the opportunities to develop novel in vitro experimental strategies that might substitute standard biochemical protocols and screening technologies. For example, nitrilotriacetic acid (NTA) chelator nanoarrays have been printed by DPN on glass substrates, and they allowed selective His-tagged protein immobilization from cell extracts [89]. Ribosome display technique and DPN were combined for protein nanoarray generation from cell-free systems, a biotechnological tool in principle suitable for arraying of different proteins on the same chip without further purification [90]. In the proof-of-principle experiment, protein-ribosome-mRNA fusion molecules

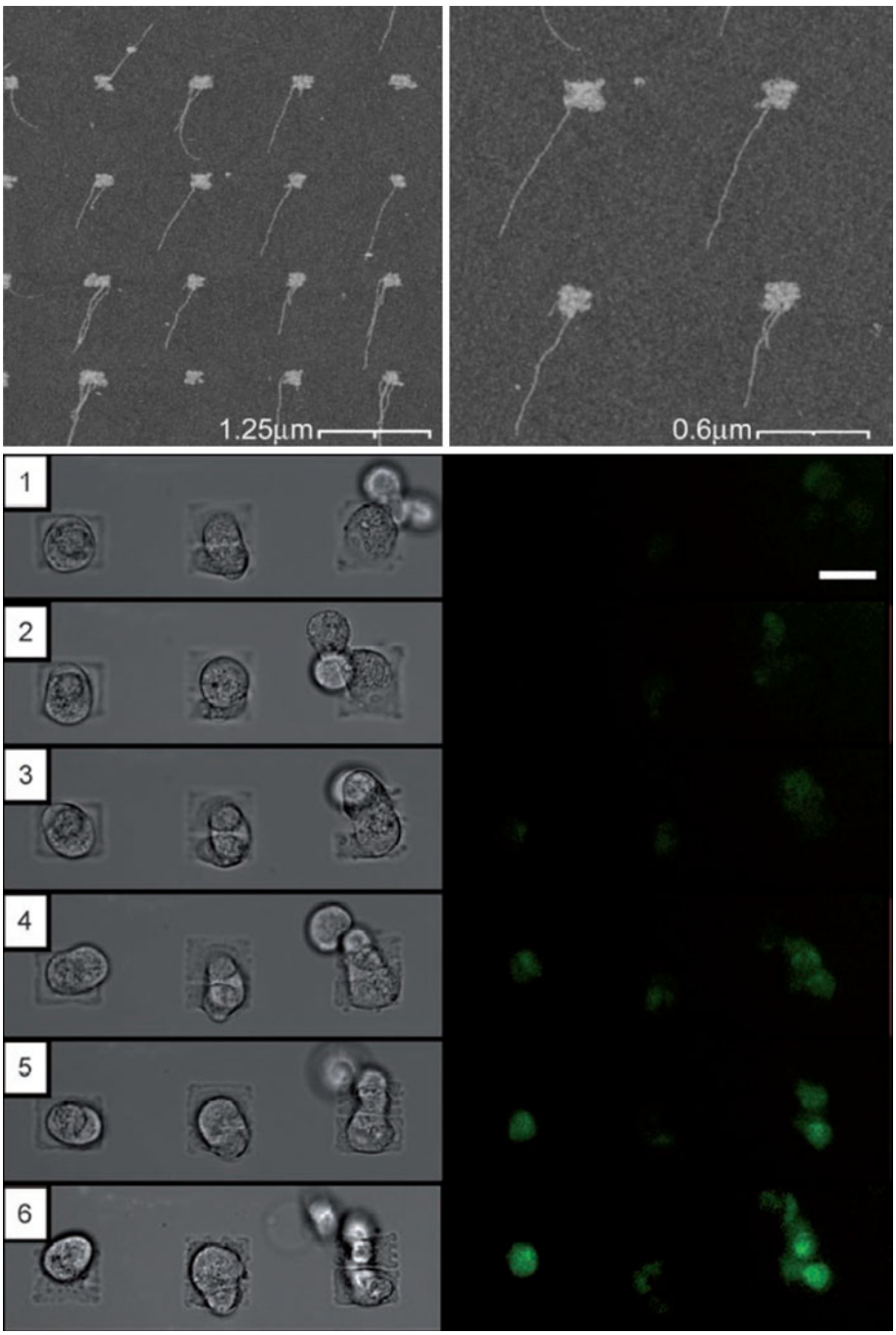

Fig. 4 Single-cell level virology on nanobiochips. *Top panel*: single filamentous M13 bacteriophage virus particles were immobilized and aligned over cm-sized surface areas by employing nanoarrays of antibodies fabricated with subtractive printing technique (reproduced from [86]). *Bottom panel*: virus infectivity of single cells. Time-lapsed DIC images (*left*) and fluorescence (*right*) images of CV1 cells infected by recombinant parainfluenza virus SV5 (the viral genome was encoded with an EGFP gene) nanoarrays. The viral array consisted of 250 nm-sized dots, spaced 1.5 µm apart (21 dots × 21 dots, 32 µm × 32 µm). Images were taken every 20 min (data shown here have a 3-h time interval between each image of 1–6, that is, 74–89 h post infection). The intensity of green fluorescence from CV1 cells on the patterns increases over time. The viral nanoarray technology, in principle, allows controlling the number of virus particles introduced to each cell (reproduced from Vega et al. [88])

were produced by coupling in vitro translated individual proteins to their corresponding mRNA. Only a desired protein conjugated to a specific RNA sequence was immobilized from the mixture of fusion molecules to complementary ssDNA spots on a nanoarray printed by DPN. Contrary to the existing microarray technologies, in similar nanobiochip platforms the amount of the biological material needed to obtain a meaningful proteomic data set in principle can be reduced to the content of several or even single cells. Establishment of such "nanoproteomic" analytical platforms might be a natural development following the already established platforms of single-cell genomics that proved particularly useful in microorganism research [91].

Conclusion and outlook

To conclude, the nanobiochip concept has definitely entered into the phase of implementation, owing to the substantial progress achieved during the past 15 years in the field of chemical and biological nanopatterning. Structured and functionalized bio-active layouts with features at the sub-

cellular scale have already demonstrated a number of advantages over the mainstream microplate and microarray technologies. For example, stimulation and detection of different cellular processes could be carried out locally, under precisely controlled conditions. This pathway may lead to the development of new biophysical experimental techniques and, in a further perspective, to instrumental manipulation and transformation of somatic and stem cells, and microorganisms. In the research of "molecular sociology" of the cell [17], nanobiochip technology might provide interesting in vitro tools for protein complex dissection, characterization, and mimetics of different enzymatic and signaling cascades. On the other hand, the degree of integration and complexity of the present nanobiochip devices is relatively low. Multiple biocomponent and/or hybrid architectures (for example, combined with nanoelectronic, plasmonic, and other photonic structures [2, 92, 93]) are still in the research phase. They are critically important for construction of integrated nanobiodevices and also for interfacing them with other analytical, diagnostic, or delivery platforms. Most likely, the coming years will see a dynamic development in this area. Devices for site-specific and time-controlled single-cell transfection or proteomic analysis are already technologically feasible and might be soon available to the life science community. The power of single-cell technologies can be nicely illustrated by a very recent single-cell amplified genome study, which revealed complex biotic interactions among previously uncharacterized marine microorganisms, with each cell undergoing distinct types of interaction [94]. However, the driving force in nanobiochip development will be the actual unmet need in the biotechnology, pharmaceutics, medicine, environment, food and safety sectors.

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