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Monitoring Single-Cell Infectivity from Virus-Particle Nanoarrays Fabricated by Parallel Dip-Pen Nanolithography**

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Since the discovery of dip-pen nanolithography (DPN),^[1,2] our group and others have been able to routinely fabricate highly miniaturized arrays of many different classes of biological entities such as oligonucleotides,^[3–5] peptides,^[6,7] proteins,^[8–13] phospholipids,^[14] and virus particles.^[15–17] These structures have been used for a variety of applications, including templates for controlling the assembly of nanoparticles into functional materials,^[18] high-sensitivity bionanodiagnostic devices,^[19] and self-assembling nanoelectronic structures.^[20] The high resolution of DPN has even facilitated the generation of chemical templates small enough to site isolate and control the orientation of individual virus particles.^[16] Although relatively unexplored, these nanoarrays enable one to carry out studies not possible with the more conventional microarray format. In principle, this includes studies of complex biological processes such as virus-cell infectivity, proliferation, and transmission at the single-virus-particle level. However, in order to explore these fundamentally important processes, arrays of functional biological nanostructures must be combined with novel methods that allow one to monitor their interactions at the nanoscale in real time.

The study of virus particles and their interactions with host cells has been an important challenge for biologists.^[21–23] A detailed understanding of the pathogenic process between a virus and an infected host cell is not only of fundamental importance but could potentially lead to new approaches for antiviral drug development. We hypothesized that one could use nanoarrays to site isolate individual and small collections of virus particles in a manner that would allow us to study cell infectivity at the single-cell, single-particle level. In principle, one could control the type of virus introduced to the cell, the density of particles, and the spatial distribution of the particles underneath the cell. This potentially would allow one to systematically study some of the critical factors that control infectivity. In practice, however, there are two barriers to these types of single-cell assays. The first pertains to the activity (or lack thereof) of the virus particles after immobilization on the features that constitute a nanoarray, and the second concerns how one can effectively monitor the cell infection process. Herein, we report a novel approach to study and monitor virus infectivity at the single-cell level, where the number of virus particles introduced to each cell, in principle, can be controlled through the use of DPN-generated nanoarrays (Scheme 1). We show that when antibodies are immobilized on Zn^{II}-carboxylate-rich nanopatterns they can be used to assemble viruses in an active state, and cell infectivity can be monitored by using fluorescence and an enhanced green fluorescent protein (EGFP)^[24] expression protocol.^[25] This EGFP expression protocol is based on the use of reverse genetics to generate recombinant parainfluenza virus SV5 (rSV5-EGFP),^[26,27] in which the viral genome was encoded with an EGFP gene (see Supporting Information). Upon infection, infected cells produce the EGFP protein, thus, generating a green fluorescence signal.

With the advent of linear^[28] and larger two-dimensional^[29] (2D) arrays of cantilevers, it is now possible to use DPN to pattern features that are sub-100 nm to many micrometers in size over square-centimeter areas in a relatively short period of time (e.g., minutes). In the context of studying virus-cell interactions, this advance makes it possible to monitor how many individual cells interact with nearly identical nanopatterns composed of the same features. Therefore, one can collect a great deal of statistically significant data on similar cells while still taking advantage of the extraordinary resolution of the DPN technique.

In a typical experiment, an 18-pen array was used to pattern 250-nm-diameter dot features of 16-mercaptopentadecanoic acid (MHA, Aldrich, Milwaukee, WI) on a semitransparent gold-coated (10 nm Au and 2 nm Ti) glass cover slip (Figure 1A). Each feature within an array was spaced 1.5 μm apart and each nanoarray (21 dots $\times$ 21 dots, 32 μm $\times$ 32 μm) was spaced 42 μm apart. The size of each nanoarray (generated by each pen) was designed to accommodate a single cell. The regions surrounding the patterned dot features were then passivated by immersing the substrate in an ethanol solution of 11-mercaptopentadecylpenta(ethylene glycol) (PEG-SH) (5 mM) for 30 min followed by copious rinsing with ethanol. This results in a monolayer of PEG-SH on the unmodified portions of the substrate. Gold sub-

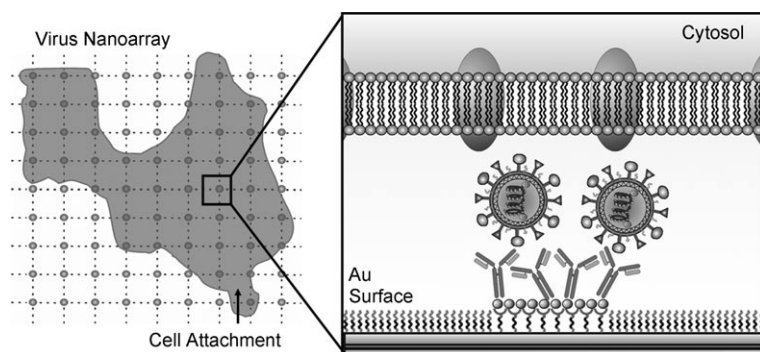
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Supporting information for this article is available on the WWW under <http://www.small-journal.com> or from the author.



Scheme 1. Schematic representation of an rSV5-EGFP nanoarray used to study single-cell infectivity.

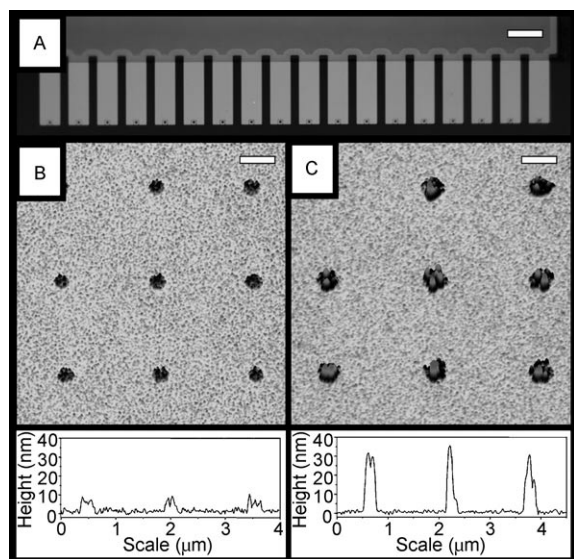


Figure 1. A) Optical image of an E-18 cantilever array. Scale bar = 77 μm . B) TM-AFM topography image and height profiles of anti-SV5 (polyclonal rabbit IgG) immobilized on MHA dot arrays pre-treated with $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$. C) TM-AFM topography image and height profile of the anti-SV5 nanoarray after the addition and capture of rSV5-EGFP particles. All AFM images were taken at the same location at a scan rate of 0.5 Hz (silicon cantilever, spring constant = 40 N m^{-1}). Scale bar = 500 nm.

strates coated with PEG-SH are known to be resistant to the nonspecific adsorption of proteins, virus particles, and cells.^[30] The substrates were then immersed in an ethanol solution of $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (5 mM, 99%, Fluka, Milwaukee, WI) for 6 h. This resulted in the immobilization of Zn^{II} ions on the carboxylic acid-rich nanofeatures.^[31,32] Residual metal ions were removed by rinsing the substrate with ethanol, followed by drying under a stream of N_2 . We have previously shown that antibodies can be effectively immobilized on such features.^[33]

In a sterile environment, a solution of the polyclonal rabbit antibody (1 mg mL^{-1}) raised against SV5 in PBS buffer (50 mM phosphate buffer, pH 7.0, 150 mM NaCl) was applied to the nanopatterned substrate and allowed to incubate for 2 h at room temperature in an air-tight humidity chamber (TeleChem International, Sunnyvale, CA). Excess antibodies were then removed by carefully washing the sub-

strates with PBS ($\approx 5 \text{ mL}$). The antibody arrays were then incubated with an rSV5-EGFP viral solution (25 μL , ca. 5×10^6 virus particles mL^{-1}) for 2 h at 37°C . The resulting virus nanoarrays were rinsed repeatedly with PBS (6 $\times$ 1 mL) to remove any nonspecifically bound viruses. The resulting virus-particle nanoarrays were characterized by tapping-mode atomic force microscopy (TM-AFM). Upon

virus binding to the features within the array, the height of a typical feature increases from $8.1 \pm 1.5 \text{ nm}$ to $31.1 \pm 2.0 \text{ nm}$ (Figure 1B and C). This observation is consistent with our previous report using metal-ion affinity templates for the immobilization of antibodies that captured enveloped virus particles on surfaces.^[33] Substrates containing the assembled virus nanoarrays were then immediately submerged into a Dulbecco's modified Eagle's medium (DMEM) solution (supplemented with 10% fetal bovine serum (FBS, Harlan, Indianapolis, IN) and 1% penicillin/streptomycin (P/S, Invitrogen, Carlsbad, CA)) to preserve their biological activity.

Cell-infection experiments were conducted by adding freshly trypsinized CV1 cells (African green monkey kidney cell; in 1 mL DMEM (also supplemented with 10% FBS and 1% P/S), ca. 2.5×10^5 cells mL^{-1}) on top of the substrates containing virus nanoarrays placed in six-well dishes (Becton Dickinson). The solution of cells and virus nanoarrays was left in an incubator (37°C , 5% CO_2 , Thermo Electron, Waltham, MA) for 1.5 h to allow for cell spreading and adherence to the patterned area. After carefully rinsing the substrates to remove unbound cells (by washing three times with 2 mL DMEM solution (2.5% FBS and 1% P/S, without phenol red)), they were placed into a glass-bottom cell-culture dish (50 mm, MatTek, Ashland, MA) with 2 mL media solution (DMEM without phenol red (Invitrogen), supplemented with 2.5% FBS and 1% P/S). The culture dish was then transferred onto a mini cell incubator (Zeiss, Thornwood, NY) mounted on an inverted fluorescence microscope (Zeiss Axiovert 200) equipped with temperature- and CO_2 -control units. Zeiss AxioVision software was used to conduct time-lapse experiments and take images in Nomarski (differential interference contrast (DIC)) and fluorescence (green channel) modes every 20–30 min. DIC and fluorescence microscopy images were taken directly from serum-containing DMEM solution (without phenol red), and the live cells were imaged in real time during all experiments. The attachment of CV1 cells on the arrays was confirmed during the first 20 h post infection (Figure 2). These CV1 cells exhibited a high affinity towards the virus nanoarrays, and at least one CV1 cell was attached to each one of the eighteen arrays. After 20 h, the majority of cells that were initially nonspecifically bound to the substrate detached and were subsequently removed by washing. The cells that remained attached to the arrays

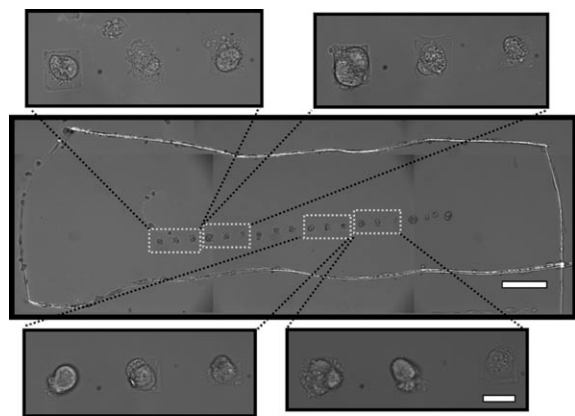


Figure 2. DIC images of assembly of CV1 cell(s) on eighteen rSV5-EGFP nanoarrays (fabricated by parallel DPN) after 20 h (post infection). The rectangular box around the boundary is the scratched alignment mark used to locate the patterned area containing the virus particle nanoarrays. The scale bar is 150 μm for the larger image and 35 μm for the insets.

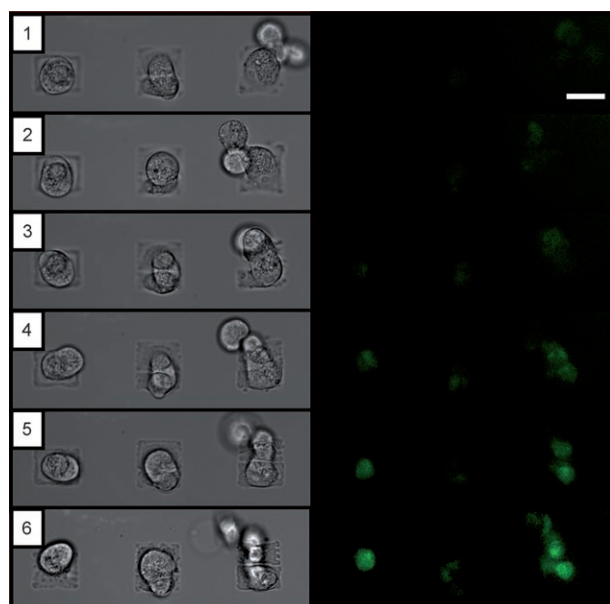


Figure 3. Time-lapsed DIC images (left) and fluorescence (right) images of CV1 cells infected by rSV5-EGFP nanoarrays (images were taken every 20 min (data shown here have a three-hour time interval between each image of 1–6, that is, 74 h–89 h post infection)). The intensity of green fluorescence from CV1 cells on the patterns increases over time.

started to show a detectable, above-background, green fluorescence signal (Figure 3) 74 h post infection (see movies in Supporting Information). This result demonstrates that the virus particles anchored on the antibody arrays had retained their biological activity. Interestingly, infection was also accompa-

nied by a change in the morphology of the attached cells from a square (wide spanning and firmly anchored to the surface) to a rounder shape, which is indicative of abnormal cell function (see Supporting Information).^[34] After 95 h post infection, 14 out of the 18 anchored CV1 cells screened were identified as being infected with rSV5-EGFP based on fluorescence measurements.

To demonstrate that the EGFP production from the cells did not occur because of an infection from nonspecifically adsorbed virus particles, a control experiment was set up using a gold substrate functionalized with only a PEG-SH monolayer and exposing it to antibodies and viruses under the same experimental conditions used for the virus nanoarrays described previously. After incubating the substrates with freshly trypsinized CV1 cells, in separate wells, fluorescence microscopy and flow-cytometry techniques were used for end-point inspection of viral infections. After 75 h post infection, patterned substrates showed an intense green fluorescence signal, whereas substrates lacking the patterned chemical templates did not exhibit any measurable green fluorescence signal (Figure 4A and B). DAPI (blue dye) was added as a nuclear staining agent so that both healthy and infected cells could be visualized and differentiated. The percentage of cells infected with the patterned substrates was determined by measuring the EGFP production using flow cytometry. Two independent runs suggested that more than 90% of the cell population was infected with rSV5-EGFP and exhibited green fluorescence one to three orders of magnitude higher than in the negative control experiments (Figure 4C). The absence of fluorescence signal from the cells exposed to the unpatterned PEG-SH passivated substrates confirms that the cell infection must have occurred specifically from the patterned virus particles and that PEG-SH is effective in minimizing adsorption of virus particles outside of the patterned areas.

In summary, we have demonstrated the ability to generate patterns of biologically active virus particles through the use of DPN-generated affinity templates. Single cells adhere to these arrays, and the virus particles are capable of infecting the cells, despite the fact that the features are submicrometer and the viruses have been immobilized by antibodies. Through the use of genetically engineered EGFP-expressing virus particles, we have developed a novel and useful assay for following the infectivity process on nanoarrays by fluorescence. Given the fact that DPN arrays have been shown to allow the precision placement of individual

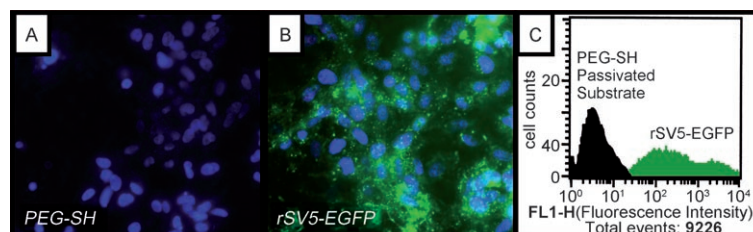


Figure 4. Fluorescence images (overlay of DAPI and EGFP) of CV1 cells exposed to PEG-SH passivated gold substrates (A) and to rSV5-EGFP nanoarrays (B). C) Quantification of the percentage of rSV5-EGFP infected cells determined by flow cytometry.

viruses on a substrate,^[16] this work, when combined with previous capabilities, will open the door for using nanoarrays to study many fundamentally important microbiological events, such as how the number of virus particles, their orientation, and their chemical immobilization and presentation affect cellular infectivity for single and small collections of virus particles.

Keywords:

cell infection • dip-pen nanolithography • templating • patterning • viruses

- [1] R. D. Piner, J. Zhu, F. Xu, S. H. Hong, C. A. Mirkin, *Science* **1999**, 283, 661.
- [2] D. S. Ginger, H. Zhang, C. A. Mirkin, *Angew. Chem.* **2004**, 116, 30; *Angew. Chem. Int. Ed.* **2004**, 43, 30.
- [3] L. M. Demers, D. S. Ginger, S. J. Park, Z. Li, S. W. Chung, C. A. Mirkin, *Science* **2002**, 296, 1836.
- [4] M. Z. Liu, N. A. Amro, C. S. Chow, G. Y. Liu, *Nano Lett.* **2002**, 2, 863.
- [5] A. Bruckbauer, L. M. Ying, A. M. Rothery, D. J. Zhou, A. I. Shevchuk, C. Abell, Y. E. Korchev, D. Klenerman, *J. Am. Chem. Soc.* **2002**, 124, 8810.
- [6] J. Hyun, W. K. Lee, N. Nath, A. Chilkoti, S. Zauscher, *J. Am. Chem. Soc.* **2004**, 126, 7330.
- [7] H. Z. Jiang, S. I. Stupp, *Langmuir* **2005**, 21, 5242.
- [8] K. B. Lee, S. J. Park, C. A. Mirkin, J. C. Smith, M. Mrksich, *Science* **2002**, 295, 1702.
- [9] K. B. Lee, J. H. Lim, C. A. Mirkin, *J. Am. Chem. Soc.* **2003**, 125, 5588.
- [10] J. M. Nam, S. W. Han, K. B. Lee, X. G. Liu, M. A. Ratner, C. A. Mirkin, *Angew. Chem.* **2004**, 116, 3110; *Angew. Chem. Int. Ed.* **2004**, 43, 1246.
- [11] J. H. Gu, C. M. Yam, S. Li, C. Z. Cai, *J. Am. Chem. Soc.* **2004**, 126, 8098.
- [12] D. Lehnert, B. Wehrle-Haller, C. David, U. Weiland, C. Ballestrem, B. A. Imhof, M. Bastmeyer, *J. Cell Sci.* **2004**, 117, 41.
- [13] H. Jung, C. K. Dalal, S. Kuntz, R. Shah, C. P. Collier, *Nano Lett.* **2004**, 4, 2171.
- [14] S. Lenhart, P. Sun, Y. Wang, H. Fuchs, C. A. Mirkin, *Small* **2007**, 3, 71.
- [15] J. C. Smith, K. B. Lee, Q. Wang, M. G. Finn, J. E. Johnson, M. Mrksich, C. A. Mirkin, *Nano Lett.* **2003**, 3, 883.
- [16] R. A. Vega, D. Maspoch, K. Salaita, C. A. Mirkin, *Angew. Chem.* **2005**, 117, 6167; *Angew. Chem. Int. Ed.* **2005**, 44, 6013.
- [17] C. L. Cheung, J. A. Camarero, B. W. Woods, T. W. Lin, J. E. Johnson, J. J. De Yoreo, *J. Am. Chem. Soc.* **2003**, 125, 6848.
- [18] L. M. Demers, S. J. Park, T. A. Taton, Z. Li, C. A. Mirkin, *Angew. Chem.* **2001**, 113, 3161; *Angew. Chem. Int. Ed.* **2001**, 40, 3071.
- [19] K. B. Lee, E. Y. Kim, C. A. Mirkin, S. M. Wolinsky, *Nano Lett.* **2004**, 4, 1869.
- [20] S. W. Chung, D. S. Ginger, M. W. Morales, Z. F. Zhang, V. Chandrasekhar, M. A. Ratner, C. A. Mirkin, *Small* **2005**, 1, 64.
- [21] R. J. Coelen, *World J. Microbiol. Biotechnol.* **1997**, 13, 365.
- [22] S. C. Harrison, B. Alberts, E. Ehrenfeld, L. Enquist, H. Fineberg, S. L. McKnight, B. Moss, M. O'Donnell, H. Ploegh, S. L. Schmid, K. P. Walter, J. Theriot, *Proc. Natl. Acad. Sci. USA* **2004**, 101, 11178.
- [23] Y. Lee, J. Yin, *Nat. Biotechnol.* **1996**, 14, 491.
- [24] B. P. Cormack, R. H. Valdivia, S. Falkow, *Gene* **1996**, 173, 33.
- [25] J. S. Sarma, E. Scheen, S. H. Seo, M. Koval, S. R. Weiss, *J. Neurovirology* **2002**, 8, 381.
- [26] B. He, R. G. Paterson, N. Stock, J. E. Durbin, R. K. Durbin, S. Goodbourn, R. E. Randall, R. A. Lamb, *Virology* **2002**, 303, 15.
- [27] B. He, R. G. Paterson, C. D. Ward, R. A. Lamb, *Virology* **1997**, 237, 249.
- [28] K. Salaita, S. W. Lee, X. F. Wang, L. Huang, T. M. Dellinger, C. Liu, C. A. Mirkin, *Small* **2005**, 1, 940.
- [29] K. Salaita, Y. Wang, J. Fragala, R. A. Vega, C. Liu, C. A. Mirkin, *Angew. Chem.* **2006**, 118, 7378; *Angew. Chem. Int. Ed.* **2006**, 45, 7220.
- [30] C. Pale-Grosdemange, E. S. Simon, K. L. Prime, G. M. Whitesides, *J. Am. Chem. Soc.* **1991**, 113, 12.
- [31] G. B. Deacon, R. J. Phillips, *Coord. Chem. Rev.* **1980**, 33, 227.
- [32] J. F. Hicks, F. P. Zamborini, R. W. Murray, *J. Phys. Chem. B* **2002**, 106, 7751.
- [33] R. A. Vega, D. Maspoch, C. K.-F. Shen, J. J. Kakkassery, B. J. Chen, R. A. Lamb, C. A. Mirkin, *ChemBioChem* **2006**, 7, 1653.
- [34] J. C. Gallego-Gomez, C. Risco, D. Rodriguez, P. Cabezas, S. Guerra, J. L. Carrascosa, M. Esteban, *J. Virol.* **2003**, 77, 10606.

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