Live-cell microarray surface coatings supporting reverse transduction by adeno-associated viruses

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High-throughput live-cell microarray technologies that facilitate combinatorial screening of genes and RNA interference (RNAi) would be invaluable in the identification of key gene expression profiles involved in complex cellular behaviors. Each spot on such a microarray can comprise a unique combination of genes or RNAi packaged into gene delivery vectors. Live target cells seeded on top of the microarrays would express the combination of genetic factors, potentially leading to phenotypic changes within cells. Here, we investigate the feasibility of using adeno-associated virus (AAV) as a gene delivery agent for such live-cell genetic microarrays. A robotic spotter was used to deposit AAV onto gamma-aminopropyl silane, amine silane, or nitrocellulose-coated glass slides. Virus deposition and reverse transduction of target cells were found to be surface coating–dependent with nitrocellulose coating yielding the best AAV deposition, while also producing discrete islands of highly transduced cells. Our results demonstrate the feasibility of using nitrocellulose-coated surfaces for the development of AAV-based genetic microarrays.
its delivery efficiency can be improved. Additionally, AAV is able to deliver genes into both dividing and nondividing cells. A number of naturally occurring serotypes of AAV have been identified, with each serotype displaying variable tropism (9). Numerous rational (10,11) and combinatorial (12,13) methods have been developed to enhance gene delivery efficiencies into target cells. Furthermore, AAV is nonpathogenic, with approximately 80% of the human population being seropositive for AAV2 without any known consequences (14). Recombinant AAV does not integrate effectively into human chromosomes, alleviating concerns over insertional oncogenesis. Due to these numerous positive features, we have initiated the development of AAV-based live-cell genetic microarrays.

To create AAV-based microarrays, a custom-built robotic spotter was used to deposit AAV serotype 2 (AAV2) encoding GFP (AAV2-GFP) onto glass slides (Figure 1). For the microarray to be successful, AAV needed to be localized into spots, but maintain the ability to release from the surface and transduce target cells. If virus affinity to the surface is too low, virus patterns may dissipate quickly upon addition of cell seeding media, resulting in a lack of localized gene expression profiles. Conversely, if affinity to the surface is too high, vectors may adsorb irreversibly or possibly even denature, resulting in suboptimal gene delivery efficiencies. No previous reports exist testing AAV for microarray development; therefore, we screened several commonly used surface coatings [gamma-amino propyl silane (GAPS), amine silane, and nitrocellulose] to determine an optimal substrate for AAV immobilization and reverse transduction.

**Materials and methods**

**AAV production**

A triple transfection method using HEK 293T cells was used to create AAV2-GFP as described previously (15). Virus was purified using ultracentrifugation and a step iodixanol gradient. Virus used in these experiments was purified using a heparin affinity column with dialysis against Dulbecco’s phosphate-buffered saline (DPBS), as virus in iodixanol inhibited deposition from the array pin. Virus solution (1 mL) was added to a heparin column (GE Healthcare, Piscataway, NJ, USA), rinsed with column buffer (150 mM NaCl, 10 mM Tris, 10 mM MgCl₂), and eluted with elution buffer (1 M NaCl, 10 mM Tris, 10 mM MgCl₂). Eluted virus was added to a 10,000 molecular weight cut-off dialysis cassette (Pierce, Rockford, IL, USA) and dialyzed against three changes of DPBS over 9 h. Virus was titered using real-time quantitative PCR (RT-QPCR) with primers against the cytomegalovirus (CMV) promoter in the recombinant virus cassette.

**Robotic spotting**

The robotic spotter utilized three independent, high-precision motorized stages purchased from Zaber Technologies (Vancouver, BC, Canada). A 300-mm travel linear slide was used for all x-axis spotting translations, as well as movements between a megasonic wash station (Arrayit, Sunnyvale, CA, USA), a 384-well plate containing samples to be spotted, and the spotting surface. A 13-mm travel linear stage was used to translate the spotting surface along the y-axis, and a 28-mm travel linear actuator was used to raise and lower the printhead and pin. The entire robot was enclosed by plexiglass for humidity control. The robot spotter was controlled through an RS-232 connection using custom LabView software (National Instruments, Austin, TX, USA) incorporating VIs provided by Zaber Technologies. Software inputs the number of rows, columns, and samples, as well as the center-to-center pitch between spots and starting coordinates of the array. The software then serially controls each of the three motorized stages through four sequential operating modes: sample pickup, spotting, washing, and drying.

**Virus spotting**

GAPS-coated slides (Corning, Corning, NY, USA) and amine silane–functionalized slides (CEL Associates, Pearlard, TX, USA) were used as is. These slides should yield similar surfaces, but with different groups linking to the glass substrate. Nitrocellulose (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in methanol at concentrations of 2–4 mg/mL. Glass slides were rocked for 1 h with nitrocellulose solutions at room temperature and then allowed to dry at room temperature for 1 h. Spotting was conducted using an in-house fabricated microarray robot (see previous Robotic spotting section) and an SMP15 pin (7 nL delivery volume) from Arrayit. Slides were allowed to dry for 1 h, backfilled with StabilGuard (Surmodics, Eden Prairie, MN, USA) for 30 min, and finally rinsed with PBS.

**Immunostaining**

Slides were blocked with 1% BSA-PBS for 1 h, rinsed with PBS, and then incubated with a 1:200 dilution of A20 antibody (American Research Products, Belmont, MA, USA) at 4°C overnight. Microarrays were then rinsed with PBS and incubated with a 1:100 dilution of a goat-anti-mouse AlexaFluor 532 (AF532)-conjugated antibody (Invitrogen, Carlsbad, CA, USA) for 1 h. Samples were washed with PBS and mounted using Fluoromount.

**Cell culture**

Slides were seeded with HeLa cells in a 10-cm tissue culture dish. GAPS- and amine-functionalized slides were seeded at a confluency of 95%, and nitrocellulose slides were seeded at a confluency of 20%. Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen). Slides were moved to a new dish the next day and supplemented with fresh media. Slides were fixed with 4% paraformaldehyde (PFA) in PBS 72 h post cell-seeding. Cell viability was determined using the LIVE/DEAD assay (Invitrogen) with 4 μM EthD-1 and 2 μM Calcein AM in DPBS. Samples were incubated for 45 min and imaged.

**Imaging and analysis**

Microarrays were imaged using a Zeiss LSM 5 LIVE confocal microscope (Carl Zeiss, Munich, Germany). Integrated densities were determined using ImageJ. Data are reported as average with standard error of the mean. Statistical significance was determined using analysis of variance (ANOVA) and Tukey’s Honestly Significant Difference (HSD) for...
posthoc pairwise testing \((P < 0.05, \text{JMP 9 software; SAS, Cary, NC, USA}).\)

**Results and discussion**

Deposition of AAV on the three different surfaces was visualized via immunostaining with an antibody that detects non-denatured AAV2 capsids only (Figure 2, A–C). Spot diameter for all three cases is approximately 400 μm with a pitch of 800 μm, as expected. Although GAPS and amine silane coatings allow for localized AAV deposition, the density of non-denatured virus deposition is low, highlighted by low fluorescence values on the intensity plots (Figure 2, D–E). Low levels of AAV2 adsorption on amine surfaces is consistent with our previous studies (16). Here, edge effects can be observed where more virus is deposited along the edges of the spots. In contrast, the nitrocellulose coating yields bright virus staining, indicating a greater amount of non-denatured AAV is immobilized in the spotted area (Figure 2F). Our previous work indicates a hydrophobic methyl [−CH₃] surface is able to mediate high levels of AAV2 adsorption (16), suggesting the hydrophobic character of nitrocellulose may support more effective AAV immobilization. Interestingly, a higher distribution of AAV is observed along the center axis of the tip, likely attributable to the geometry of the microarray pin. The pin used in this study has a linear slit opening positioned at the center of the tip, and the sample presumably flows out of the slit opening and spreads along the shape of the tip.

Next, we seeded HeLa cells on top of the virus patterns to test for reverse transduction. Here, immobilized AAV must release from the surface and be internalized by cells in order to mediate successful gene delivery. This release cannot happen too quickly, otherwise virus patterns will be dispersed as they come into contact with cell media. Although GAPS- and amine silane-coated slides result in positively transduced cells, the GFP-expressing cells are scattered and do not yield distinct patterns (Supplementary Figure S1). The non-specific affinity of AAV to these surfaces may not be strong enough to resist disassociation in media prior to cell attachment. In other words, as soon as cells and media are added to the virus patterned slides, viruses may lift off and drift away from the established patterns. The nitrocellulose-coated slide, however, successfully yields a pattern of GFP-expressing cells (Figure 3A), indicating AAV is able to both remain adsorbed to the surface even in the presence of cell culture media and effectively release from the surface to be internalized into cells. Notably, cells are attached only to these discrete spot regions, with no cells adhering to the background (Supplementary Figure S2). We observe a series of linear cell islands reflective of the higher AAV deposition along the center axis of the microarray pins (Figure 2C). We hypothesize that cells may be able to utilize specific receptor-ligand interactions to adhere to this region displaying higher amounts of AAV. AAV2 is known to bind heparan sulfate proteoglycans on target cell membranes (17). At sufficient concentrations, immobilized AAV2 may be able to attract and bind cells, as previously demonstrated (18). In future optimizations, adding cell adhesive proteins to the virus solution could help generate complete spot formation. Alternatively, we may use a different pin shape to obtain a more uniform virus deposition.

We next sought to optimize the promising nitrocellulose coating by testing different nitrocellulose concentrations (Figure 3B). Preliminary tests indicated coatings with >4 mg/mL nitrocellulose are not efficient for gene delivery, while <1 mg/mL leads to undesirable virus spreading (Supplementary Figure S3). We hypothesize that higher concentrations of nitrocellulose may lead to undesirable high levels of virus entanglement, resulting in inefficient release of the virus from the substrate. In a concentration range of 2–4 mg/mL nitrocellulose, we observe similar levels of GFP expression in cells. Cell viability is also maintained on 3 mg/mL nitrocellulose, as compared with tissue culture plastic (Supplementary Figure S4). We also investigated the minimum concentration of virus needed to obtain significant gene expression on the nitrocellulose surface. Using a 3 mg/mL nitrocellulose-coated substrate, we spotted a range of $5 \times 10^{10}$ to $1 \times 10^{13}$ viral genomes (vg)/mL AAV solutions. A sharp decrease in gene expression was observed when a virus solution under $5 \times 10^{11}$ vg/mL is used (Figure 3C). Based on a spot size of 400 μm and a pin deposition volume of 7 nL, we estimate a virus concentration of $3 \times 10^{12}$ vg/mL is required to create a monolayer of virus. Due to inefficiencies in the system, we likely do not have a monolayer, and not all AAV that is bound is able to release effectively and deliver genes into target cells.

In summary, our AAV-based live-cell genetic microarray platform represents a promising alternative to platforms using other viruses. We have identified an appropriate nitrocellulose substrate that is capable of tightly localizing AAV deposition, unlike alternative substrates that show virus spreading and a lack of
gene expression localization. AAV has been previously shown capable of modification to improve gene delivery efficiencies and specificities. Moreover, AAV has a greater safety profile compared with other viruses, supporting its continued use and investigation in gene delivery applications. Finally, we have demonstrated that AAV can be patterned using a robotic spotter and that immobilized AAV is capable of releasing from the nitrocellulose surface and mediating effective reverse transduction.

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**Competing interests**

The authors declare no competing interests.

**References**


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