Reports

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-amino propyl 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.

Live-cell genetic microarray technology is a potentially powerful approach for investigating the effects of expressing or knocking down various combinations of genes on cellular behavior (1). Each spot on the microarray can contain a different combination of genes or RNA interference (RNAi) cassettes, upon which live target cells can be seeded. Through the process of reverse transduction (when viral vectors are used) or reverse transfection (when nonviral vectors are used), cells will internalize the genes or RNAi cassettes resulting in either the overexpression or knockdown of the target gene, respectively. Different expression profiles for each microarray spot may lead to phenotypic changes in the cells. Detecting these changes via automated imaging and image processing will provide a high-throughput combinatorial screening methodology that could aid in the identification of key gene expression profiles that yield a desired cellular outcome.

Live-cell genetic microarrays have previously been generated using both viral and nonviral gene delivery methods (2–4). Nonviral gene delivery has been used with single-cell microarrays to study up to 600 cell spots on a single chip (5). Small inter-

fering RNA (siRNA) lipoplexes mixed with Matrigel have been successfully arrayed onto poly-L-lysine-coated slides for high-throughput siRNA silencing (6). The success of any live-cell genetic microarray approach, however, hinges upon the ability of vectors to deliver nucleic acids with high efficiency to cells of interest. Although nonviral delivery systems are promising, viral vectors in general still have significantly higher gene delivery efficiencies, supporting their continued development for genetic microarray technology. In terms of viral vectors, both adenovirus and lentivirus have been spotted under various conditions to create genetic microarrays. For example, lentivirus was spotted with a robotic spotter to create alternating patterns of overexpression and short hairpin RNA (shRNA) cassettes, and phenotypic changes in target cells were analyzed (3). Fibrin hydrogels have been used to help immobilize lentiviruses in spot patterns, suggesting a difficulty in maintaining lentivirus spots without such an approach (7). Adenovirus microarrays have been created using a manual spotting device and 25 mg/mL nitrocellulose-coated slides (4). Notably, a synthetic blocking agent was used to localize cell adhesion to areas of virus

deposition, creating distinct cell islands. Such studies made great strides toward the creation of versatile, high-throughput genetic microarray technology, and we sought to improve upon these successes through the use of an alternate, potentially more advantageous gene delivery vector.

Specifically, additional improvements in delivery efficiency and safety would enhance the potential for virus-based live-cell genetic microarrays. A virus vector capable of being modified relatively easily to increase transduction specificity and efficiency would increase the power of this technology. Thus, we chose to investigate the potential of adeno-associated virus (AAV) for use on genetic microarrays. AAV is a 25 nm icosahedral parvovirus that is currently being tested in 75 human clinical trials. The simple viral genome of AAV is composed of only two genes: (i) *rep* encodes for nonstructural replication proteins and (ii) *cap* encodes for three structural proteins and an assembly-activating protein (8). The structural proteins self-assemble to form the supramolecular capsid.

AAV is an appealing alternative to the other viruses due to its increased safety profile and the relative ease with which

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

Virus 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,

Figure 1. Schematic of patterning process. Recombinant AAV is spotted onto coated glass slides. Slides are backfilled with microarray stabilizer and then seeded with cells. Arrays are imaged 3 days later for gene expression.

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 spotter

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, Pearland, 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 spotter section) and an SMP15 pin (7 nL delivery volume) from Arrayit. Slides were allowed to dry for 1 h, backfilled with StabliGuard (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. Intensity plots were created using ImageJ [National Institutes of Health (NIH), Bethesda, MD, USA]. Lines were drawn through an image of four spots, and the fluorescence intensity profile was plotted as a function of distance in microns.

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

Figure 2. Immunostaining of AAV deposition on three different coated glass slides. Virus was spotted onto (A) GAPS slides, (B) amine silane slides, or (C) 3 mg/mL nitrocellulose-coated slides. Samples were immunostained with an anti-AAV2 antibody and an AF532-conjugated secondary antibody. Scale bar, 500 μ m. (D–F) Images are intensity plots generated over four spots using ImageJ. Bars at top of graphs indicate locations of virus patterns. While patterned deposition is observed on all surfaces, the most intense staining can be seen with the nitrocellulose-coated slides.

posthoc pairwise testing ($P < 0.05$, 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 nondenatured AAV2 capsids only (Figure 2, A–C). Spot diameter for all three cases is approximately 400 μ m with a pitch of 800 μ m, as expected. Although GAPS and amine silane coatings allow for localized AAV deposition, the density of nondenatured 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 nondenatured AAV is immobilized in the spotted area (Figure 2F). Our previous work indicates a hydrophobic methyl ($-\text{CH}_3$) 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 spot, 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 nonspecific 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 undesirably 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 5×10^{10} to 1×10^{12} viral genomes (vg)/mL AAV solutions. A sharp decrease in gene expression was observed when a virus solution under 5×10^{11} vg/mL is used (Figure 3C). Based on a spot size of 400 μ m and a pin deposition volume of 7 nL, we estimate a virus concentration of 3×10^{11} 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

Figure 3. Cell adhesion and gene expression on nitrocellulose-coated slides. AAV was printed on nitrocellulose-coated glass slides, and HeLa cells were seeded on top of the virus patterns. (A) Pattern of GFP-expressing cells can be observed on 3 mg/mL nitrocellulose-coated slides at 72 h post cell-seeding. Cells are located only within the patterns. Scale bar, 500 μ m. (B) Nitrocellulose coatings (2–4 mg/mL) yield similar levels of reverse transduction. The differences are not statistically significant. (C) Different concentrations (virus genomes/mL) of AAV were used in the spotting and efficiency of reverse transduction quantified. A dose-dependent response in gene expression is observed, with significantly less gene expression observed with virus at concentrations of 5×10^{10} and 1×10^{11} vg/mL ($P < 0.05$).

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.

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