A Microfluidic Platform for High-Throughput RNAi Screening

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SAND2011-5075P

INTRODUCTION

High-throughput, genome-wide RNAi screens are an effective method of identifying the set of host factors that are vital to viral infection and reproduction; however, current implementations of the technology are expensive and rely on bulky equipment. **We are currently developing a portable, low-cost microfluidic platform capable of rapidly and accurately performing high-throughput RNAi screens in top-level biocontainment facilities.**

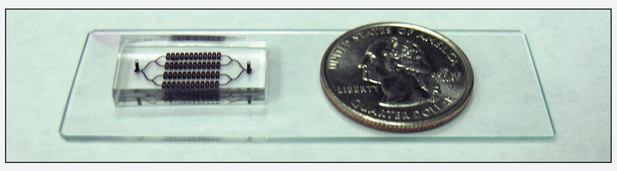


Figure 1. A 96-well diffusion-type microfluidic RNAi screening device sits on a glass slide. Cell culture, reverse transfection, viral infection and staining all take place within the device.

BACKGROUND

The key to mitigating the threat of viral pathogens lies in identifying the specific host proteins that a virus exploits for infection and reproduction. The subset of genes involved wanes in comparison to the tens-of-thousands of genes present in a typical host cell and is specific to each virus species/host cell pairing. Consequently, it is necessary to employ high-throughput approaches to systematically repress individual genes and determine the effect on viral pathogenesis. Current methods rely on either a microtiter or microarray platform to exploit the RNA inteference (RNAi) pathway that is present in eukaryotic host cells. RNAi is a process in which cellular machinery uses small RNAs (e.g. siRNAs) to repress the expression of corresponding genes. Virologists can use RNAi to knock down a specific gene in a cell culture and then assess the effect on the ability of a virus to infect the modified cells. In a high-throughput screen (HTS), each well or spot can contain a different type of siRNA, so a multitude of genes can be screened simultaneously. Most of the current HTS RNAi technologies rely on bulky robotics to facilitate the preparation of these high-diversity samples.

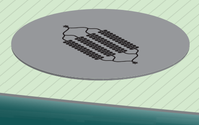
ADVANTAGES OF MICROFLUIDICS

	Microfluidics	Microarray	Microtiter
Capital Cost	Low	High	High
Reagent Use	Low	Low	High
Cross-Contamination	Medium	High	Low
Bench Space Requirement	Low	Medium	High
Difficulty of BSL-3/4 Use	Low	High	Medium

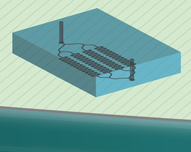
Microfluidic RNAi screening produces results that are comparable to larger screening platforms at a fraction of the cost and time. The total volume of a single microfluidic screening well is 0.2-1 nL, whereas a microtiter well requires 100-300 µL of reagents. The microfluidic channel design mitigates cross-contamination issues associated with the microarray platforms. Unlike microarray and microtiter plate platforms, this microfluidic platform does not require bulky robotics and is capable of advanced preparation for easy and rapid transition into BSL-3/4 labs, requiring no specialized equipment beyond a fluorescence microscope and common micropipettes.

METHODS

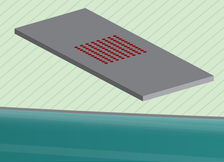
Master Fabrication
Masters are fabricated via soft lithography. A negative photoresist is spin coated onto silicon wafers to a final thickness of 25 µm. A mask is used to cover the wafer, leaving only the area of the design to be exposed to UV light. During the development process, areas of photoresist that were not exposed to UV light are removed.



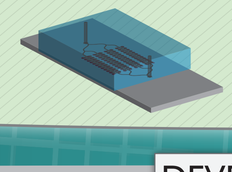
Device Fabrication
Completed masters are placed in the bottom of petri dishes, covered in a 0.5-1 cm thick layer of PDMS, and then baked to allow the PDMS to harden. The cooled device is cut out using a razor, and inlet and outlet holes are fashioned using a 750 µm biopsy punch. If the device is to be used for cell culture, it is autoclaved for sterilization.



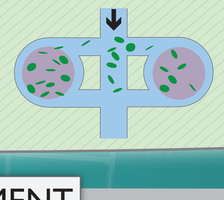
Reverse Transfection Mix Spotting
Reverse transfection mix containing siRNA (for RNAi), lipofectamine (a transfection agent), gelatin (hardening compound), and water is spotted onto a GAPS II coated slide. The siRNA type can vary by spot, by column or by row. Spotting is accomplished either by hand (using a microarray spotting device), or by using a microfluidic spotting device.



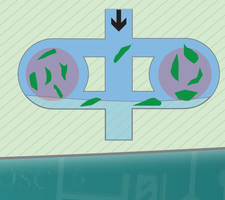
Device Alignment & Preparation
Devices are aligned to the spots using a dissection microscope. At this point, assembled devices can be stored for up to 6 months before use. Prior to loading, devices may optionally be placed under vacuum to mitigate the formation of bubbles as fluid fills the wells.



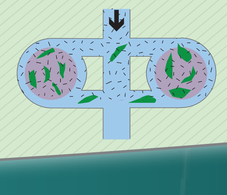
Cell Loading
Cells are loaded into the device using a standard micropipette. Approximately 10 µl of cell solution is injected into the inlet. After the device is fully loaded, the top (including the inlet and outlet) is covered with 1 mL of media, to arrest any persistent flow within the device and to prevent bubbles from forming in the inlet or outlet.



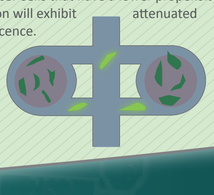
Media Profusion
To insure cell viability, the media inside the device must be exchanged daily. The amount of media required for complete displacement varies by device. Manual media exchange is performed using a micropipette; however, a novel gravity perfusion system can provide continuous flow for up to 48 hours.



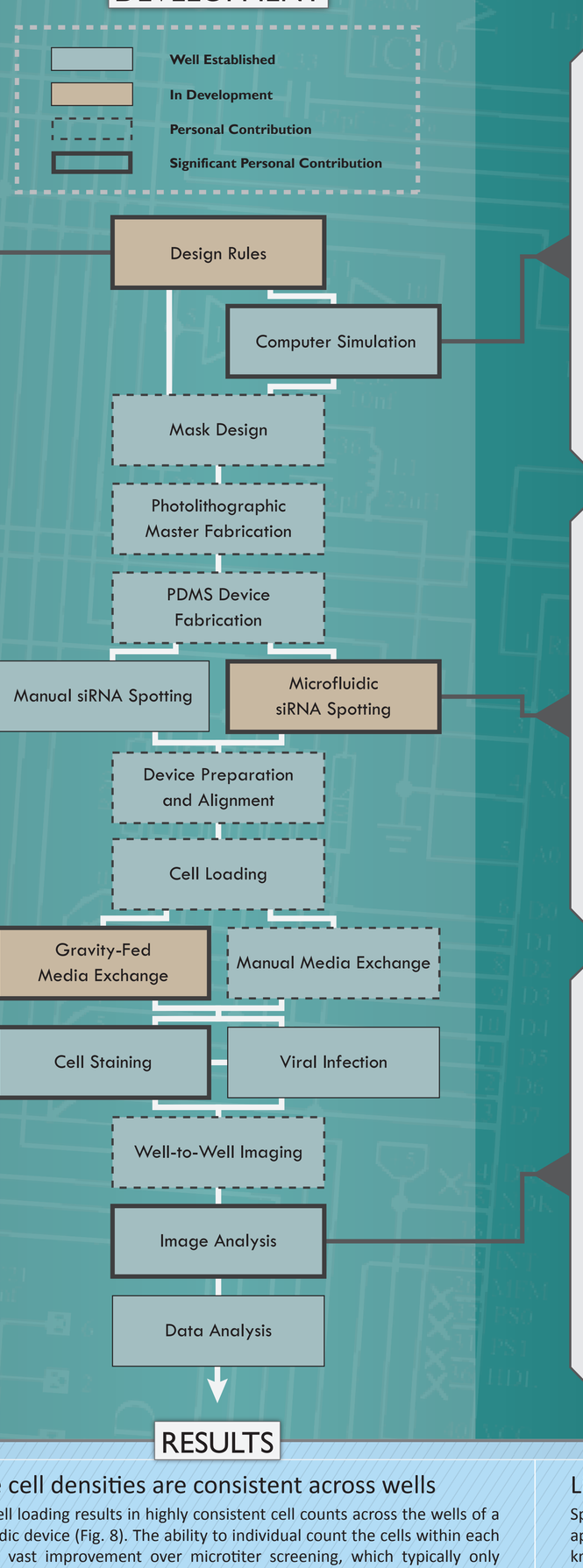
Virus Infection
After the cells have incubated for 24 hours, solution the virus (in this case, Rift Valley Fever Virus) is injected into the device, allowed to incubate for 1 hour (during which time the virus infects cells) and is then flushed from the device.



Fluorescence Microscopy
'One day after virus infection, fluorescence microscopy is used to assess the impact of the RNAi knockdown on viral infection. Cells that have been infected by a GFP-modified virus will fluoresce. Cells that have a lower propensity for infection will exhibit attenuated fluorescence.



DEVELOPMENT



DESIGN RULES

Development of the RNAi microfluidic platform is governed by 3 underlying principles:

1. Simplicity: The device must be simple to fabricate and use. It must require minimal specialized equipment to facilitate its dissemimation into BSL-3/4 labs and general wet labs.

2. Low Cost: The per-device and per-run costs must be minimal. Devices must be able to be prepared in advance and stored for extended periods of time prior to use.

3. Efficacy: The platform must provide a level of performance comparable or superior to competing technologies while adhering to priciples 1 + 2, above.

Current device iterations fulfill the design rules by adapting basic cell culture and RNAi concepts to a microfluidics workflow. The designs avoid the use of multi-layer fabrication, valve systems, and equipment like syringe pumps to minimize the complexity associated with fabrication and utilization. siRNA spotting and cell culture conditions are optimized to accomodate for simple operation while mitigating problems like cell death and siRNA cross-contamination.

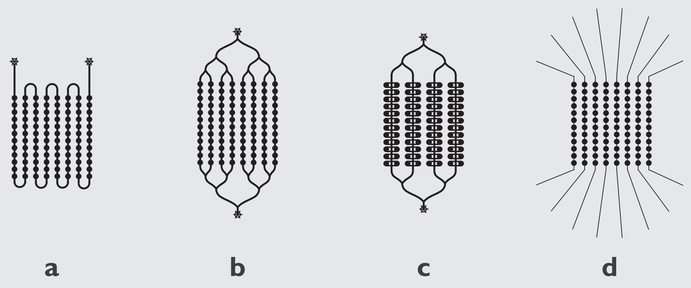
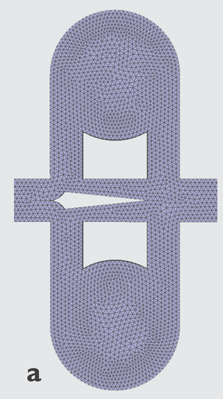


Figure 3. Current device designs: (a) inline series device, (b) split inline parallel device, (c) split diffusion device, (d) microfluidic spotting device (used to spot reverse transfection mix samples in replicate by column). All devices feature 600 µm diameter wells and 150 µm lines (with the exception of the spotting device, which has 75 µm lines).

COMPUTER SIMULATION



The analysis of new designs and device features is accomplished through the use of an open-source workflow combining FreeCAD modeling and Elmer multi-physics simulation. A directional loading/profusion system was designed for the diffusion-type device using Elmer to optimize the fluidics (Fig. 2). The in-channel element allows for differential cell loading and media profusion (one direction increases flow into the wells for cell loading, whereas the other decreases flow to prevent cells from being rinsed from the wells).

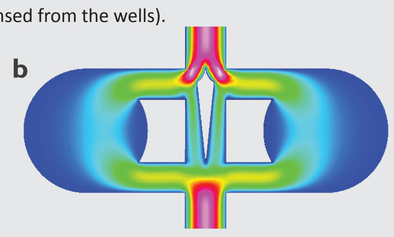


Figure 2. (a) Elmer mesh and (b) velocity profile from the directional diffusion well simulation.

MICROFLDUIDIC SIRNA SPOTTING

Currently, reverse transfection mix spotting is accomplished using expensive printers or through tedious manual process. Both methods produce inconsistent spots in terms of volume and concentration. As an alternative approach, a microfluidic spotting platform has been developed to cut down on time and cost while improving the consistency of spots. The current device (Fig. 3d) is capable of producing an array of 96 spots in replicates of 12. Each column is addressed with a dedicated inlet/outlet system, minimizing cross-contamination. Microfluidic spotting requires 15 minutes of labor and around 12 hours of drying time, a dramatic improvement over the tedious 4 hours of labor (and 12 hours of drying time) that manual spotting requires.

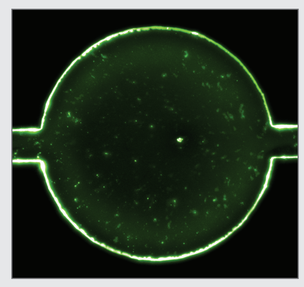
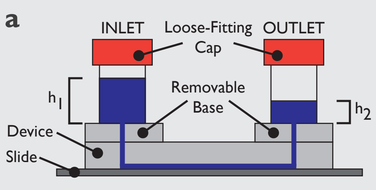


Figure 4. A 600 µm wide spot of reverse transfection mix (gelatin, lipofectamine, water, and green fluorescing siRNA) on a GAPS II coated glass slide after drying and removal of spotting device.

GRAVITY-FED MEDIA EXCHANGE



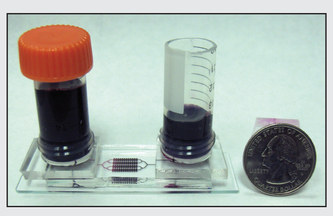


Figure 5. (a) Diagram and (b) photo of a device with a gravity-fed media exchange system.

One of the inherent problems with microfluidic cell culture systems is the buildup of waste and rapid depletion of nutrients within the wells. Continuous media profusion is the ideal approach to mitigating the problem of media stagnation. We have developed a gravity-driven system that allows for continuous media profusion for up to 48 hours without user intervention. The flow rate of media profusion can be precisely controlled by varying the difference in initial volume of the two reservoirs. Staining solution and virus particles can be injected through the reservoir holes or by removing the reversibly sealed bases.

IMAGE ANALYSIS

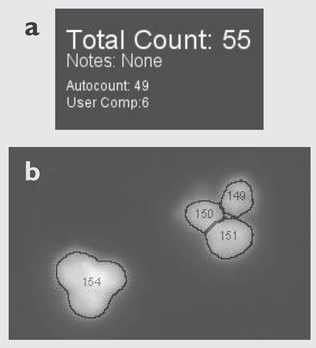


Figure 6. (a) Count detail overlay from a processed image. (b) A clump of cells (left) missed by the watershed function.

During the development process, it is important to rapidly and effectively determine the number of living and dead cells within each well. While automated cell counting methods exist, they are often inaccurate, especially when analyzing images of confluent cells. As such, a hybrid macro was developed for ImageJ, allowing user intervention to correct exposure, check the automated count, and manually compensate for any errors. The macro then overlays the count information over the top-left corner of the image and saves the file as a compressed JPEG, with the count also recorded in the filename. Previous counting methods required about 8 hours per device, whereas the macro accomplishes equally accurate counts in 30 minutes.

Microfluidic spotting is effective for RNAi

Spots resulting from microfluidic spotting are more consistent in form and concentration across replicates than comparable spots spotted by hand (Fig. 7c) or printing by machine (data not shown). Microfluidic spotting is a viable and superior alternative to these methods when efficiency and consistency across replicates is a high priority.

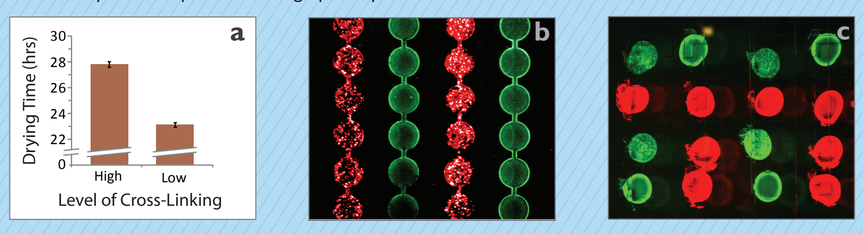


Figure 7. (a) Lower levels of cross-linking increase drying time (n=3, error bars=SE). (b) Alternating red and green fluorescent siRNA spotted with a microfluidic device (Fig. 3d). (c) A pattern of red and green fluorescent siRNA spotted by hand.

Viable cell densities are consistent across wells

Simple cell loading results in highly consistent cell counts across the wells of a microfluidic device (Fig. 8). The ability to individual count the cells within each well is a vast improvement over microtiter screening, which typically only quantifies well fluorescence, requiring additional controls to quantify the cells.

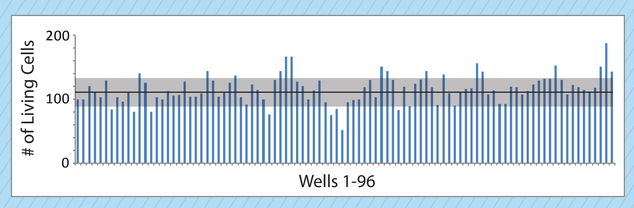


Figure 8. Per-well cell counts for an inline parallel control device (no siRNA spotting prior to loading) after 48 hrs of incubation. Grey area is one standard deviation (+/- 22.4) and the black line is the average (115.9).

Lipofectamine 2000 exhibits cytotoxicity

Spotting concentrations of Lipofectamine 2000 used for microarrays are too high for microfluidic applications, resulting in high levels of transfection mix cytotoxicity. In the experiment below, siRNAs known to be toxic do not appear to induce higher levels of toxicity than Lipofectamine spotted without siRNA (Fig. 9). Scrambled siRNA (CNT) and no spotting (Empty) have lower cell death.

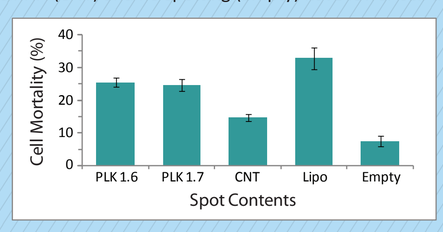


Figure 9. HeLa cell mortality as a function of reverse transfection in a microfluidic device (n=12, error bars=SE). PLK1.6/PLK1.7, known to be toxic, permeate cells at the same rate as Lipofectamine alone (Lipo). CNT mix contains scrambled siRNAs that should not interact with any genes in HeLa cells.

CONCLUSIONS AND CONTINUED DEVELOPMENT

> We have developed a 96-well microfluidics system capable of 72-hour cell culture and high-throughput RNAi

> Our tube-based gravity-fed profusion system is a simple substitute for manual media exchange or complex, multi-layer reservoirs

> Our novel microfluidic spotting system is a promising alternative to manual spotting

>> A new device design will introduce direction-specific loading and profusion, improving loading density and minimizing cross-contamination

>> The concentration of transfection agent in the spotting solution will be refined to minimize cytotoxicity

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