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Dynamic Gene Expression Profiling Using a Microfabricated Living Cell Array

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We describe the development of a microfluidic platform for continuous monitoring of gene expression in live cells. This optically transparent microfluidic device integrates high-throughput molecular stimulation with nondestructive monitoring of expression events in individual living cells, hence, a living cell array (LCA). Several concentrations of a soluble molecular stimulus are generated in an upstream microfluidic network and used to stimulate downstream reporter cells, each containing a green fluorescence reporter plasmid for a gene of interest. Cellular fluorescence is continuously monitored and quantified to infer the expression dynamics of the gene being studied. We demonstrate this approach by profiling the activation of the transcription factor NF-κB in HeLa S3 cells in response to varying doses of the inflammatory cytokine TNF-α. The LCA platform offers a unique opportunity to simultaneously control dynamic inputs and measure dynamic outputs from adherent mammalian cells in a high-throughput fashion. This approach to profiling expression dynamics, in conjunction with complementary techniques such as DNA microarrays, will help provide a more complete picture of the dynamic cellular response to diverse soluble stimuli.

Technologies such as Northern blots and reverse transcription polymerase chain reaction have been extensively applied to the monitoring of gene expression in cells and tissues for numerous biological investigations. Recent developments in DNA microarray technology have further expanded the scope of these investigations by enabling the simultaneous monitoring of several genes.[1−4] While these techniques provide snapshots of changes in gene expression at single time points, they are not ideal for investigating time-dependent behavior. Often, to approximate continuous-time measurements, multiple cell populations are destructively measured at several discrete time points. A technique allowing continuous, nondestructive monitoring would complement current genomics technologies by providing a more dynamic picture of gene expression. Green fluorescent protein (GFP) technologies[5] have recently emerged to allow noninvasive measurements of cell function and cell responses;[6−8] however, GFP-based expression studies are typically performed in traditional single-dish or multiwell formats to monitor a small number of stimulation conditions.

Microfluidics is an inherently scalable technology, offering a unique opportunity to simultaneously screen a wide range of experimental conditions with relative ease (different concentrations, combinations, and temporal profiles of molecular inducers, inhibitors, and modulators). Poly(dimethylsiloxane) (PDMS) microfluidic devices[9,10] are ideal for cell-based applications involving fluorescent protein expression because of their well-established biocompatibility and optical transparency. They have been applied to several biological studies including chemotaxis,[11] cell and small-molecule patterning,[12,13] cell sorting,[14,15] and biochemical separations.[16] Recent advances in cell-based microfluidics have enabled adherent mammalian cells to be cultured in microchannels for days to weeks;[17] however, functional measurements such as gene expression have not been demonstrated.

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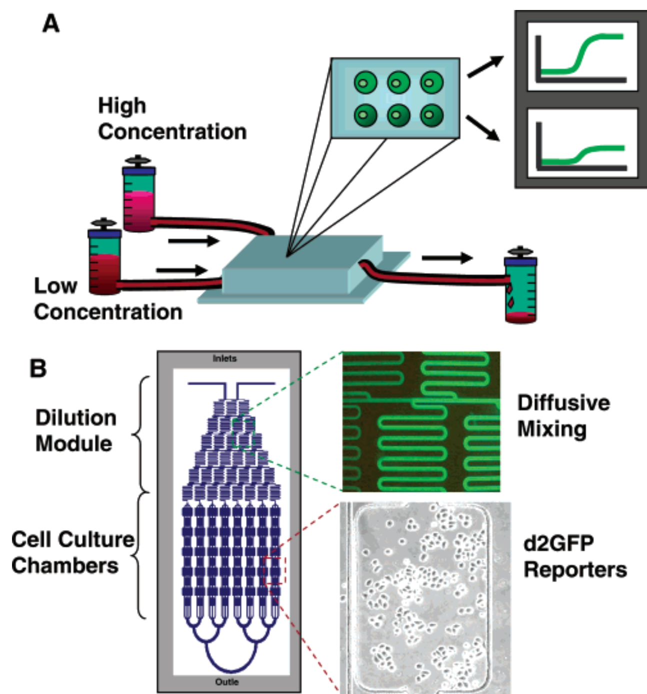

Figure 1. Living cell array (LCA) (A) Schematic representation of the LCA device. Medium containing a soluble mediator enters the device, delivers nutrients, stimulates cells cultured in the cell chambers, and exits into a waste stream. Gene expression dynamics are obtained by time-lapse imaging and quantified using image analysis software. (B) The microfluidic network design and micrographs of the dilution and cell cultivation modules are shown. The transparent PDMS device allows visualization of the TNF-α gradient (doped with fluorescein dye) and EGFP reporter cells. The microfluidic channels in the upstream dilution module are 50 μm in width and 50 μm in height and generate several concentrations of the stimulus by continuous-flow diffusive mixing of adjacent laminar flow streams. The various concentrations are delivered to the downstream array of culture chambers, each 800 μm long and 500 μm wide.

In this report, we describe the development of a functional genomics tool that combines GFP reporter technology and microfabrication for dynamic gene expression profiling. This tool, the living cell array (LCA) (Figure 1A), can be used for simultaneously stimulating and monitoring the time course of gene expression in living cells. The feasibility of studying dynamic gene expression using the LCA is demonstrated by profiling the activation of a transcription factor (NF-κB) in response to eight different doses of a cytokine (TNF-α). The resultant fluorescence of the entire population is monitored with single-cell resolution, allowing direct measurement of population dynamics and biological heterogeneity in the culture. The LCA has the potential to significantly impact investigations where various gene expression events and their interactions need to be studied in a time-dependent manner with individual cell resolution.

EXPERIMENTAL PROTOCOLS

Microfluidic Design. The LCA consists of an upstream *dilution module* that generates a range of stimulus concentrations and a downstream *cell culture module* where EGFP reporter cell lines are grown in individual chambers (Figure 1B) of a single microfluidic network. The dilution module, based on the fluidics of a microgradient generator previously described by Li Jeon et al.,[11] consists of a highly interconnected network that generates eight outlet concentrations spanning two inlet concentrations. Successive steps of diffusive mixing between adjacent laminar flow streams occur in long (50 × 75 × 10000 μm) channels to ensure complete mixing at the relevant flow rates. Each outlet stream then feeds into a downstream array of 800 μm × 500 μm cell chambers (Figure 1B).

Device Fabrication. Microchannels and cell chambers were fabricated in PDMS (Dow Corning, Corning, NY) using soft lithography and rapid prototyping techniques.[9] Inlets and outlets were drilled with a blunted and beveled syringe needle, and the resulting PDMS microfluidic network was irreversibly bonded to a glass slide assisted by oxygen plasma surface treatment (150 mTorr, 50 W, 20 s), creating a sterile optically transparent device for cell culture and gene expression profiling.

Bioreactor Design. The supporting bioreactor consisted of the microfluidic devices, medium-containing reservoirs for gravity-driven flow, and associated tubing. Inlet and outlet reservoirs were drilled and outfitted with plastic connectors (Small Parts Inc., Miami Lakes, FL). Inlet reservoirs were tapped at the bottom to allow medium outflow without air incorporation or bubble generation. Outlet reservoirs were tapped near the top to drain culture effluent without suffering back-pressure buildup. All reservoirs were outfitted with sterile filter caps to allow continuous pressure equilibration for the entire system while maintaining sterility.

GFP Reporter Cell Line. HeLa S3 cells (ATCC, Rockville, MD.) were grown in high-glucose Dulbecco's modified Eagles medium (Invitrogen, Gaithersburg, MD) supplemented with 10% bovine calf serum and 100 units/mL penicillin per 100 μg/μL streptomycin in a humidified 5% CO_2 incubator at 37 °C. Plasmid pNF-κB/CMV_{min}d2EGFP (referred to as pd2NF-κB) was constructed as described elsewhere.[18] Briefly, promoter, response element, and d2EGFP reporter gene (EGFP with a 2-h half-life) were excised from a Clontech Living Colors plasmid (Clontech, Paolo Alto, CA) and cloned into an expression vector. Cells were electroporated, selected for plasmid integration, sorted for maximum responsiveness with minimal background fluorescence,[18] and subcloned to obtain the stable reporter cell line (HeLa-NF).

Cell Seeding. Microfluidic bioreactors were autoclave sterilized, rinsed with PBS, and degassed by driving trapped air through the walls of the gas-permeable device. Fluidic networks were precoated with 50ng/mL fibronectin (Sigma Chemical) overnight to promote HeLa-NF cell attachment. Excess fibronectin was removed by rinsing with PBS, and devices were seeded with cells. A suspension of HeLa-NF cells (3 × 10^6 cells/mL) was injected through the device outlet and allowed to settle under static conditions for ~18 h. The device was aseptically connected to the remainder of the bioreactor to provide sterile gravity-driven medium flow, and the entire microfluidic reactor was moved to an incubated stage on a fluorescence microscope to perform the stimulus−response assay. During the assay, cells were continuously perfused with fresh medium (with no phenol red) at ~1.0 μL/min to replenish critical metabolites and remove potentially toxic wastes. Static control experiments were prepared by seeding 2 × 10^4 HeLa-NF cells into 12-well tissue culture dishes (Costar), and experiments were performed at ~40−60% confluency. All

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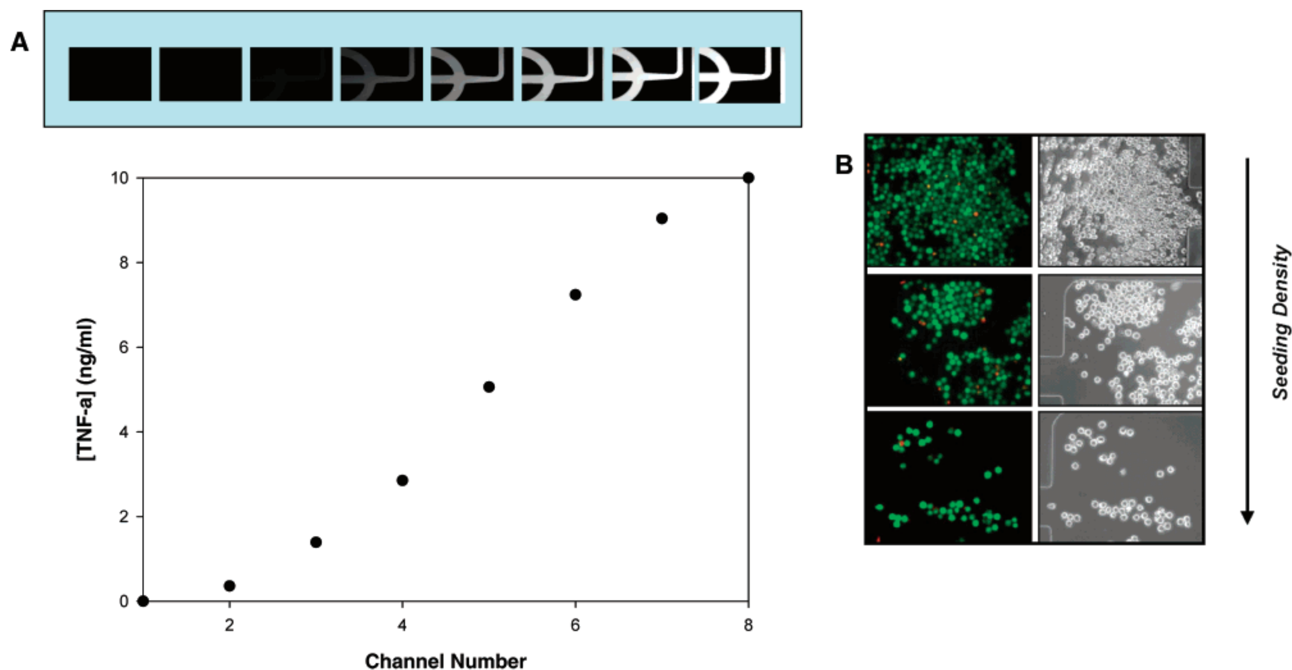

Figure 2. Characterization of the LCA dilution and cell culture modules. (A) Generation of TNF-α concentrations in the dilution module. A range of concentrations (0, 0.36, 1.39, 2.85, 5.06, 7.24, 9.04, and 10 ng/mL) of RITC-conjugated dextran (17.2 kDa) were generated in the dilution module of the LCA as described in Experimental Protocols. Fluorescence micrographs of the junction between the dilution and cell culture modules are shown. The fluorescence images were quantified and the concentration of TNF-α entering each cell culture chamber was inferred. (B) Viability of HeLa-NF cells in the cell culture chambers. HeLa-NF cells were seeded in the device at three different cell densities (1 × 10^6, 5 × 10^6, and 10 × 10^6 cells/mL) and allowed to attach in the LCA device for 24 h. Cells were stained with the LIVE/DEAD viability stain and observed on an inverted microscope with a 20× objective to assess viability. In the figure, live cells are stained green and dead cells are stained red.

experiments were performed on an incubated stage (37 °C and 5% CO_2) of a Zeiss Axiovert 200 microscope (Carl Zeiss Inc., Thornwood, NY).

Induction of NF-κB in the LCA. HeLa-NF cells were grown to ~40% confluence in either the LCA or standard tissue culture dishes and continuously stimulated with 10 ng/mL TNF-α. Phase contrast and fluorescence images were obtained every 10−60 min at preprogrammed position using a 20× objective (Carl Zeiss). Microfluidic dose−response experiments were performed by delivering growth medium containing 0.1 mg/mL RITC-dextran (Sigma) and 10 ng/mL TNF-α through one inlet and unmodified medium through the other. Inlet reservoirs were maintained at elevated heights with respect to the outlet reservoir and adjusted to achieve the desired concentrations. Continuous-flow dilutions were monitored throughout each experiment by fluorescence imaging of comparable molecular weight (17 200) RITC-dextran indicator. The total flow rate was ~1 μL/min. Phase contrast and fluorescence images were captured every 30−60 min at three positions per TNF-α concentration.

Image Analysis. Fluorescent images were analyzed using Metamorph V 6.0r4 (Universal Imaging Corp., Downington, PA). The average fluorescence of each cell region was corrected for medium and device fluorescence as well as illumination fluctuations and nonuniformities by subtracting the average local background fluorescence at each time point. The result was scaled by the region area and divided by the number of cells in the region to determine an average intensity per cell in the region. The difference between the initial and final average intensity per cell was plotted to compare the response to the various concentrations of TNF-α.

RESULTS AND DISCUSSION

Development of the LCA. We present a LCA platform that combines microfluidic solution handling with GFP reporter technology to continuously and noninvasively profile gene expression dynamics in living cells. The specific LCA device described here generates several concentrations of a soluble mediator and stimulates GFP reporter cells cultured in the device. Activation of gene expression results in the induction of fluorescence that is continuously monitored to quantify gene expression dynamics (Figure 1A). The LCA contains two components: (1) a dilution module of microfluidic channels and (2) multiple cell culture chambers integrated in a single device as shown in Figure 1B.

The dilution module of the LCA device described in this study was designed to generate eight concentrations of a soluble mediator such as the cytokine TNF-α. Fluorescent RITC-dextran (17 200) was used as a detectable indicator for the comparable molecular weight TNF-α (17 000) used in this study. The fluorescence was monitored throughout the entire dilution module to verify complete diffusive mixing. The final concentration entering the downstream cell culture chambers was monitored by fluorescence microscopy to quantify the stimulus and was found to remain steady throughout the experiment. Figure 2A shows the corresponding TNF-α concentration profile ranging from 0 to 10 ng/mL generated using the dilution module. The dilution module in the current LCA prototype can be used to generate a linear range of concentrations of any soluble molecular mediator spanning the two inlet concentrations. By varying the inlet concentration of the mediator, it is possible to precisely and reproducibly generate a range of well-defined concentrations of a

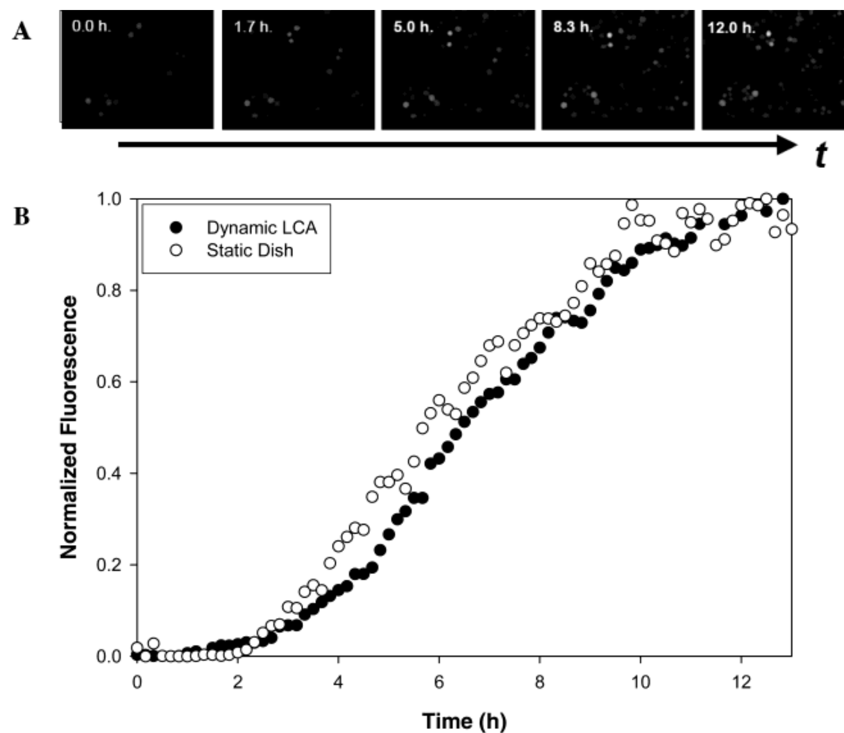

Figure 3. Induction of EGFP fluorescence in the LCA. (A) HeLa-NF cells were grown in the LCA and stimulated with 10 ng/mL TNF-α. Phase and fluorescence images were captured on a Zeiss Inverted microscope every 10 min using a 20× objective. Representative fluorescence images from a single region in the LCA are shown at various time points during the course of the experiment. (B) The temporal fluorescence profiles from TNF-α stimulated HeLa-NF cells in the LCA at a flow rate of 1.0 μL/min and in standard tissue culture dishes were determined. The time course of fluorescence was found to be similar in both culture platforms. Normalized data are shown to facilitate comparison.

soluble molecular mediator such as the cytokine TNF-α. The data presented here demonstrate the ability to generate a range of concentrations in the dilution module and deliver them to downstream preseeded cells.

Microfluidic Cell Culture. The PDMS/glass chamber was coated with fibronectin to promote cell attachment to the device. Based on previous work in our laboratory, this extracellular matrix protein maximized HeLa-NF cell attachment when compared to laminin and collagen (not shown). For the HeLa-NF cells used in this work, overnight incubation with 50−100 ng/mL fibronectin was sufficient to support uniform cell spreading and maintenance of morphology similar to that observed on tissue culture plastic. In the absence of surface modification, however, few cells attached.

Cells were introduced into the device via the inlet and allowed to attach under static conditions. Due to the large surface area-to-volume ratios in the device, a concentrated cell suspension ($(1-10) \times 10^6$ cells/mL) was required. This concentration, ~2-fold greater than conventional tissue culture protocols, allows efficient seeding by delivering a large number of cells into the channels of the small-volume device. Seeded devices were placed in tissue culture incubators for 24 h to allow for proper cell attachment and spreading prior to stimulation with TNF-α.

The suitability of the cell chambers for seeding and maintaining cells was determined using cell viability measurements. HeLa-NF cells were seeded in the LCA cell chambers as described in Experimental Protocols and allowed to grow under flow conditions for 24 h prior to viability measurements using the LIVE/DEAD stain (Molecular Probes, Eugene, OR). Our data show greater than 90% viability over a 10-fold range of cell seeding densities ($(1-10) \times 10^6$ cells/mL; Figure 2B). Furthermore, HeLa cell division was also routinely observed in the LCA cell chambers, suggesting that the microfabricated LCA provided an environment conducive to cell proliferation (not shown).

Expression Dynamics in the LCA versus Static Tissue Culture. We demonstrated the feasibility of monitoring gene expression dynamics in the LCA device by profiling the induction of NF-κB in HeLa-NF reporter cells and comparing to standard tissue culture formats. Cells were seeded in the LCA as described in Experimental Protocols and periodically monitored for 24 h to obtain a stable baseline fluorescence measurement prior to induction of NF-κB. NF-κB was stimulated in HeLa-NF cells by perfusing with medium containing 10 ng/mL TNF-α. The fluorescence profile was monitored over time and showed an increase in fluorescence relative to the baseline value (Figure 3A). In comparison, no significant fluorescence signal was observed for HeLa-NF cells in the absence of TNF-α stimulation (not shown). The fluorescence profile was quantified using image analysis and compared to that observed for HeLa-NF cells stimulated with 10 ng/mL TNF-α in a six-well tissue culture plate (i.e., static incubation). The temporal fluorescence profiles in the LCA mirrored those observed in standard tissue culture, with similar increases in fluorescence relative to unstimulated controls occurring by 2 h (Figure 3B).

Cells cultivated in the LCA experience shear stress of ~0.5 dyn/cm^2 (based on a flow rate of 1 μL/min and chamber dimensions of 500-μm width and 50-μm height) due to the continuous perfusion of medium, as compared to cells in static tissue culture formats. This value is below the range of shear

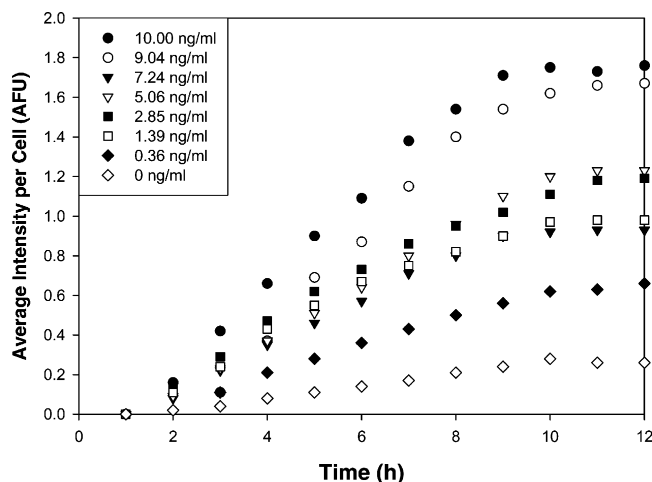

Figure 4. Dynamic profiling of NF-κB activation in the LCA. Eight concentrations of TNF-α were generated in the dilution module and used to stimulate HeLa-NF cells in the cell chambers for 2 h. TNF-α was washed away by flowing regular growth medium through the entire LCA, and the increase in fluorescence was monitored. Phase and fluorescence images were taken every 1 h for 12 h and the images analyzed using Metamorph. Background-corrected data reflect the increase in fluorescence per cell from one position in the cell chamber for each TNF-α concentration.

stress values reported to disrupt cell function.[19] This is especially important for NF-κB, as its responsiveness to shear stress is well documented.[20] The similar fluorescence profiles between the LCA and static culture suggest minimal shear induction.

Dynamic Expression Profile of NF-κB Induction in the LCA. The LCA was used to simultaneously monitor NF-κB activation in response to a range of TNF-α concentrations in a single experiment. HeLa-NF cells were seeded in the device as described earlier and simultaneously exposed to a 2-h pulse of eight different TNF-α concentrations (ranging from 0 to 10 ng/mL) generated in the dilution module. HeLa-NF cells exhibited similar fluorescence kinetics in the LCA at all TNF-α concentrations tested. The average per cell fluorescence increase observed for cells exposed to 0−10 ng/mL TNF-α is shown in Figure 4A. The magnitude of fluorescence induction appeared to be dose-dependent, with increasing fluorescence being observed in response to increasing concentrations of TNF-α. This dose-dependent response was further confirmed by stimulating HeLa-NF cells with the same concentrations of TNF-α and monitoring the induced fluorescence using flow cytometry (not shown). While fluorescence response to extreme TNF-α concentrations (high and low) was reproducible, quantitative responses to intermediate concentrations exhibited more variation due to population heterogeneity, highlighting the importance of single-cell measurements.

To our knowledge, this is the first report of dynamic gene expression measurements in a microfluidic device using adherent mammalian cells. The power of the LCA lies in its ability to simultaneously screen many conditions and efficiently explore the large parameter space relating stimulation to cell responses. The LCA has distinct advantages over conventional as well as more recently published methods for comprehensive profiling of gene expression events and has potential applications in fundamental investigations of signal transduction and gene expression. A common paradigm in cell biology is the dose-dependent interaction of molecular mediators in determining cellular responses. Roth et al.[21] have shown that the cytokines interleukin-1β and oncostatin M have either a synergistic or attenuating effect on the extracellular acidification rate of HepG2 cells depending on the doses of the two cytokines. While a thorough investigation of all such interactions can be time-consuming and tedious, the LCA would easily facilitate such combinatorial studies, including dynamic monitoring of interaction kinetics.

The use of EGFP-fusion proteins for nondestructive monitoring of gene expression has been well demonstrated by Nelson et al.,[22] who continuously monitored activation dynamics of the transcription factors STAT6 and NF-κB in living cells. While this approach provides quantitative information from living cells, it is generally performed in standard cell culture or multiwell plate format,[22] and it is therefore not ideal for testing complex combinatorial interactions of mediators and their effects on multiple expression events. Furthermore, it is often difficult to stimultaneously control stimulation and monitor changes in the cell populations using traditional platforms.

Ziauddin and Sabatini[23] recently described a high cell density microarray to study the function of several genes in a parallel format using transient transfection. Although these cell microarrays provide parallel monitoring capabilities, they are not fluidically addressable, making the simultaneous testing of several stimulators difficult. Moreover, the sensitivity of this approach is limited, as not all cell lines are equally amenable to reverse transfection.[24] The LCA addresses both these aspects by using stably transfected cells in an addressable cell array format.

One of the limitations of the method described here is the need for transcription, translation, and maturation of EGFP prior to its detection. Previous reports have indicated that EGFP has a chromophore maturation time of ~2 h,[25] a time consistent with our experiments. While the LCA cannot be used to monitor the immediate−early molecular events in real time, our results suggest that these events can be inferred from the induced fluorescence dynamics. Another potential limitation of the LCA is that only genes that are upregulated upon stimulation can be monitored using this approach, and additional fluorescence reporter schemes (such as fluorescence resonance energy transfer) are required for monitoring changes in the expression of downregulated genes in the LCA.

The gene expression profiling capabilities of the LCA can be easily increased by adding multiple molecular mediators to the input streams in the dilution module, thereby generating eight combinations of molecular mediators. By modifying the fluidics in the dilution module, a wider concentration range of molecular

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mediators can also be tested. The flexibility of rapid prototyping for device fabrication makes it possible to further modify the current LCA prototype design to increase the number of stimulation conditions or factors tested. For one, the number of input streams can be increased to screen complex combinations of mediators in a single experiment. Similarly, it is also possible to modify the device such that more than one type of reporter cell line is cultivated in the cell chambers, thereby enabling multiple molecular events to be monitored and compared simultaneously. Additionally, the combinations of molecular mediators can be delivered according to a defined temporal profile, ranging from short pulses (~1 min) to continuous stimulation. Integration of these features into the LCA would enable complex expression profiling measurements from a small set of experiments, which would be difficult to obtain using standard tissue culture formats.

Dynamic profiling is not only limited to monitoring transcription factor activation but is also equally applicable to the study of other gene expression aspects such as promoter activity and protein−protein interactions. The incorporation of noninvasive and continuous monitoring as well as parallel processing in the LCA has significant implications for fundamental investigations of cell biology. These include applications involving drug discovery, toxicology, and biosensor development where continuous monitoring of time-dependent gene expression and efficient evaluation of multiple conditions are important.

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