A miniaturized arrayed assay format for detecting small molecule–protein interactions in cells
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Background: Two complementary approaches to studying the cellular function of proteins involve alteration of function either by mutating protein-encoding genes or by binding a small molecule to the protein. A mutagen can generate millions of genetic mutations; correspondingly, split-pool synthesis can generate millions of unique ligands attached to individual beads. Genetic screening of mutations is relatively straightforward but, in contrast, split-pool synthesis presents a challenge to current methods of screening for compounds that alter protein function. The methods used to screen natural products are not feasible for large libraries composed of covalently immobilized compounds on synthesis beads. The sheer number of compounds synthesized by split-pool synthesis, and the small quantity of individual compound attached to each bead require assay miniaturization for efficient screening.

Results: We present a miniaturized cell-based technique for the screening of ligands prepared by split-pool synthesis. Spatially defined droplets with uniform volumes of approximately 50–150 nanoliters (depending on well dimensions) are arrayed on plastic devices prepared using a combination of photolithography and polymer molding. Using this microtechnology, approximately 6,500 assays using either yeast cells or mammalian tissue culture can be performed within the dimensions of a standard 10 cm petri dish. We demonstrate that the biological effect of a small molecule prepared by split-pool synthesis can be detected in this format following its photorelease from a bead.

Conclusions: The miniaturized format described here allows uniformly sized nanodroplets to be arrayed on plastic devices. The design is amenable to a large number of biological assays and the spatially arrayed format ensures uniform and controlled ligand concentrations and should facilitate automation of assays. The screening method presented here provides an efficient means of rapidly screening large numbers of ligands made by split-pool synthesis in both yeast and mammalian cells.

Introduction
Understanding how Nature’s network of cellular components carries out cellular functions is a challenging goal for chemists and biologists. The genome sequencing projects currently under way are already providing valuable information relevant to this challenge. The yeast genome has been fully sequenced [1] and it is projected that the human genome will be completed by 2005 [2]. The compilation of genetic sequence data alone will not elucidate the functional relevance of the newly identified gene products. The next challenge will be to understand how the proteins encoded by the genes function within the milieu of a living system. Achieving this goal requires the ability to alter protein function.

Classical genetics has provided the predominant means of altering protein function, resulting in the activation or inhibition of proteins through genetic mutations. For example, inactivation can occur if the mutation abrogates a protein–protein interaction critical for cell signaling, whereas activation can occur if the mutation facilitates a new interaction. Particularly useful are conditional mutations, where the inactivating effect is only observed under ‘non-permissive’ conditions accessible to the experimenter.

Whereas the genetic approach refers to the manipulation of genes as an indirect means of altering protein function, ‘chemical genetics’ refers to a conceptually analogous approach of elucidating cellular processes using small molecules rather than genetic mutations. In this case, small molecules target proteins directly to modify their activity. There are many known examples of small-molecule natural products that bind specifically to target proteins and alter their function. The immunosuppressant rapamycin and the
Detrantsforming agent trapoxin, for example, bind to and inactivate FKBPs12-rapamycin-associated protein FRAP and histone deacetylase (HDAC), respectively [3,4]. Recent investigations have taken advantage of the conditional inhibitory activity of these natural products to gain insight into the functions of these proteins within our respective signaling pathways [5-7]. A small-molecule ligand can also provide a useful handle on the protein of interest, and facilitate its purification or the identification of homologs with similar activity.

The classical genetic and the chemical genetic approaches are analogous in what they achieve, but there are cases where one approach will be more suitable than the other. Extensive conservation of protein function between yeast and mammalian cells has made yeast a useful genetic system for studying the function of mammalian proteins. There are, however, processes in mammalian cells, such as apoptosis and differentiation, that have no biochemical counterparts in yeast. Studying such pathways using conventional genetics is time-consuming and difficult. The small-molecule approach to altering protein function within cells allows for transient and conditional perturbation of a cellular system and can therefore provide a powerful tool for studying such complex mammalian processes, even in the context of whole organisms. This type of control of protein function creates a more direct link between chemistry, biology and medicine.

Classical genetics has proven so instrumental because it provides the general ability to explore protein function in a wide variety of biological processes. The practical ease with which large numbers of mutations can be generated and assayed for their effects on function is an attractive feature of this approach. In yeast, for example, millions of mutations can be generated by using a mutagen or retrotransposon. Since tens of thousands of yeast cells can be plated on an agar dish, it is feasible for a single person to screen a large library of mutations within a typical benchtop workspace. Identification of the specific mutation with the desired effect can also be accomplished: the mutated gene can be propagated in Escherichia coli, extracted and sequenced. Upon identification, the gene can be mutated in the respective host, allowing further examination of the protein it encodes.

Although natural products have facilitated an understanding of many cellular processes, a more complete set of ligands will be required in order to make the ligand-based approach completely general. One strategy for doing so uses solid-phase split-pool synthesis, which can generate millions of compounds [8,9]. (For an application of this approach to the discovery of 15 SH3 ligands from a collection of 1.1 million compounds, see [10].) Encoding schemes [11-13] are now available that simplify the structural characterization of a biologically active compound. There remains a demand, however, for effective screening strategies that will accommodate millions of ligands made by split-pool synthesis.

Conventional screening of small molecules is often performed using automated systems that conduct assays typically in a 96-well plate format. This method has been useful for screening natural product libraries or synthetic libraries of limited complexity. This ligand-discovery strategy, however, is expensive, with high costs for consumables, and it is not practical for screening many millions of compounds. Libraries of ligands on beads impose additional constraints on the format of a biological assay. These libraries can be screened with the compound still attached to the bead (on-bead assays), typically by assaying for binding of a target protein to the bead [14,15]. In this case, spatial segregation is inherent in the assay since each bead has a unique ligand attached to it. One limitation is that small molecules covalently attached to beads often do not interact with proteins in the same way as they do when free in solution [16]. Cell-based assays using released compounds have the advantage of a built-in filter against those ligands that are unable to permeate the cell. This selects for the discovery of molecules that can be used to study protein function in vivo in a conditional manner, in the same way that temperature-sensitive mutants of a gene can be used to study the protein of interest. Cell-based assays can also be designed with considerable flexibility, allowing a wide range of biological processes to be investigated. Off-bead assays are, however, limited in that each bead in the library typically contains 100 pmol or less of a unique compound; consequently, there is a requirement for assay miniaturization in order to achieve sufficient concentrations to screen individual beads for desired effects. Furthermore, since the released ligands diffuse rapidly, there is a requirement to segregate spatially the test cells in such a way that the segregants can be exposed to the effects of individual beads.

In the previous paper [17], we described a technique for performing miniaturized cell-based assays in randomly generated nanodroplets with a range of volumes. Here, we present a miniaturized assay format for detecting small molecule-protein interactions in cells within uniform and arrayed volumes of the order of 50-150 nl. The large-scale screening of ligands synthesized by split-pool synthesis in a variety of cell types can be achieved using this technique.

Results

Photolithographic preparation of PDMS templates
Photolithography using a high-resolution transparency as a mask [18] produces ‘master’ plates (Figure 1) against which plastics, in this case, polydimethylsiloxane (PDMS) can be molded to create arrays of dimple-like indentations or wells with 1 mm diameters and depths of either 40 or 150µm (19,20); R.J.J., D.C. Duffy, E. Ostuni, N.D. Willmore and G.M.W., unpublished observations). PDMS is
The photolithographic procedure used to prepare master templates and the resulting plastic PDMS plates used to prepare arrayed nanodroplets. The wells are 1 µm in diameter with a depth of either 40 or 150 µm, and an interwell spacing of 250 µm. In the wetting step, a mixture of cells and beads are pipetted onto the plate. Upon dewetting excess liquid is removed to yield nanodroplets within the wells.

poured over the master plates, cured at 65 °C for 4 h to cross-link the polymer and then peeled off the master. Cells, media and beads can be deposited in the array of wells by a simple and mild wetting–dewetting procedure. The surface of the molded PDMS is wetted with the cell and bead mixture (see below), then the excess liquid is pipetted off during the dewetting procedure. Nanodroplets of uniform volume remain within the wells upon dewetting owing to surface tension, thereby providing a formatted grid of spatially defined, miniaturized assays (R.J.J., D.C. Duffy, E. Ostuni, N.D. Willmore and G.M.W., unpublished observations). With 250 µm spacing between the wells, this format yields a dense array of miniature assays such that approximately 6,500 cell culture assays can be performed within the dimensions of a standard 10 cm diameter plastic dish (Figure 2).

Wetting and dewetting with beads
Each individual bead from an encoded split-pool synthesis will have a unique ligand and several encoding tag molecules for ligand identification. Beads used for split-pool synthesis are approximately 80 µm in diameter, and their distribution within the droplets depends on the concentration of beads used. Wetting–dewetting a suspension containing 10 mg of beads per ml of 0.006% agar in water results in a distribution of the number of beads per well. Approximately 18% of the wells remain empty, 44% contain one or two beads, 18% contain three beads, and the remaining wells contain four or more beads. More dilute bead mixtures result in most wells containing a single or a couple of beads per well (Figure 3), but with an increase in the number of empty wells. More concentrated bead mixtures result in nearly all wells containing multiple beads per well.

Depositing fibronectin in the wells for growth of adherent cells
Using the wetting–dewetting procedure with a solution of fibronectin followed by evaporation of the resultant nanodroplets, PDMS can be pre-coated with fibronectin to facilitate attachment and growth of adherent mammalian cells within the droplets. The autoclaved plastic devices were wetted and dewetted with a solution of 0.5 mg/ml fibronectin. The fibronectin was distributed within the droplets and allowed to adsorb to the bottom of the wells for 2 h as the nanodroplets evaporated. Following deposition of fibronectin, the wells were washed three times with PBS to remove excess fibronectin. Following fibronectin pre-treatment, adherent cells can then be grown onto the fibronectin-coated wells. The viscosity of tissue culture medium, which typically contains 10% fetal bovine serum, necessitates one alteration of the wetting–dewetting procedure. Upon wetting the surface with cells, a 'squeegee' action is performed across the PDMS with a glass coverslip to dewet and form droplets within the wells. The adherent cells settle within the droplets and attach to the deposited fibronectin. A pool of media is quickly pipetted into the petri dish to surround the PDMS
The visualization of arrayed nanodroplets. A saturated solution of brilliant green dye in triethylene glycol was used for visualization of a PDMS plate with spatially segregated, uniform volumed nanodroplets. There are approximately 6,500 droplets (50 nl each) per plate as shown, nanodroplet erra-

Ligand-induced growth inhibition of yeast cells

In the preceding paper, it was shown that yeast cells will replicate in droplets of 50–200 nl in volume and that small molecule-dependent selection can be used as a basis for detection of a small molecule–protein interaction [17]. In this study, an initial experiment related to the generic screen for cell division control genes (cdc screen) was performed to test the reliability of detecting ligand effects in arrayed 50 nl droplets containing yeast and beads in media. In this test system, the temperature-sensitive mutant alleles of the cdc screen were replaced with small molecules. Previously, using a deconvolution approach, a ligand that inhibits yeast growth was selected from a collection of 125,000 small molecules prepared by split-pool synthesis (J. Morken, J. Huang and S.L.S., unpublished observations). This molecule was attached to beads via a photocleavable linker and mixed with yeast cells in liquid media (compare with [17]). Using the wetting–dewetting procedure, the bead and yeast mixture was distributed over the plastic to form droplets, at a density such that some droplets contained beads and yeast while other droplets contained only yeast. At this point, the yeast cells were barely visible owing to the small number of yeast cells originally used, estimated to be 100 cells per nanodroplet. In order to release the compound from the bead, the droplets were irradiated with long wavelength ultraviolet (UV) light (365 nm) and incubated overnight to allow yeast cells to undergo multiple replicative cycles. Following UV irradiation, it was observed that wells without beads contained easily observable (by microscopy with a charge-coupled device [CCD] camera) microcolonies of growing yeast cells. In comparison, all droplets that contained a bead displayed obvious visual evidence of yeast growth inhibition. The ligand effect was restricted to the well that contained the bead, which demonstrates the absence of diffusion between droplets or through the plastic (Figure 4a). To verify that the photoreleased ligand gave a specific yeast inhibition effect, droplets containing beads with the same ligand and yeast were left untreated with no UV irradiation. As shown in Figure 4b, microcolonies of yeast cells were observable after the incubation period in wells with and without beads. This experiment demonstrates that the inhibition of yeast growth is a specific effect of the released ligand and that the bead with the immobilized ligand is itself inert.

Ligand-induced apoptosis of mammalian cells

An attractive feature of the arrayed technique is the mildness of the wetting–dewetting procedure. The mild conditions relative to the stochastic nanodroplet technique [17] enable assays to be performed with even the most sensitive mammalian cells. Since the plastic in which the cells are grown is transparent, these mammalian cell cultures can be screened visually, for example by observing morphological alterations, apoptosis, or GFP fusion protein localization. Detection of a ligand-induced effect by a visual assay was performed using mink lung cells (MvILn). The mitotic inhibitor podophyllotoxin was used because it can induce apoptosis in mammalian cells [21], a process that can be readily visualized by formation of apoptotic bodies during death [22]. Mink lung cells were first plated onto the fibronectin-coated wells and allowed to grow overnight. Podophyllotoxin attached to beads with a photocleavable linker (compare with [17]) was distributed
Within the nanodroplets, and then cleaved off the bead by UV irradiation (Figure 5a). Neither photolysis by itself nor the presence of non-irradiated podophyllotoxin beads induced an apoptotic effect (Figure 5b), demonstrating the specificity of the assay. Induction of apoptosis could be visualized by characteristic cell blebbing only in those droplets containing UV-irradiated beads (Figure 5c).

Discussion

The arrayed nanodroplet technique described in this paper allows for efficient screening of large collections of small molecules within uniform volumes of approximately 50-150 nl (depending on the depth of the well) arrayed on plastic devices. By using master plates prepared by photolithography, generation of the plastic devices is simple and straightforward. The wetting-dewetting procedure can rapidly distribute a large number of beads without individual bead manipulation. Increasing the concentration of beads yields wells with numerous beads, which increases the throughput of the assays in the initial round of screening. Although there is a limited amount of compound on a single bead (~100 pmol), only a small fraction of it is required to achieve up to high micromolar concentrations since the assay volumes are small. For example, complete release of ligand from a bead would theoretically yield a 2 mM solution within a 50 nl droplet. We typically aim to generate a 1 μM solution in initial experiments. Upon discovering a well with a desired cellular readout, the multiple beads in the well can subsequently be individually micromanipulated and re-assayed, where re-irradiation leads to the release of additional compound (A.J.Y., data not shown).

The yeast growth inhibition assay supports the conclusion that ligand-dependent yeast assays can be performed reliably in arrayed nanodroplets. It also demonstrates that the wetting-dewetting procedure can distribute yeast and beads effectively to yield consistent growth inhibition results. Simple coating of the wells with fibronectin by the wetting-dewetting procedure allows for spatial segregation of adherent mammalian cells, thereby allowing these cells to be exposed to a single or a few ligands. The podophyllotoxin-induced apoptosis of mink lung cells demonstrates that mammalian cell assays can be performed in arrayed nanodroplets. The observable phenotypes (growth inhibition or apoptosis) that are ligand specific show the ability to perform visual screens of ligands in both yeast and mammalian cells. The arrayed, spatially defined nature of the assays should make it easier to automate such screens by interfacing CCD cameras with pattern recognition software that could direct the movements of a robotic arm to sites on a grid that corresponded to specific nanodroplets.

High-density screening formats have been developed for conventional in vitro enzyme inhibition assays [23] but a key feature of the arrayed nanodroplet method is that it should be compatible with a wide variety of screens. Engineered cells that allow detection of small molecules that inhibit defined protein-protein interactions have already been used successfully in arrayed nanodroplets [24]. The generality of this format makes it amenable to the readouts (or slight modifications of them) typically used to study biological systems, as a means of detecting small molecule-protein interactions. For example, cells could be engineered to monitor expression of a reporter gene as a downstream readout to screen for small molecules that can activate or inhibit a protein important for an upstream signaling event, or fluorescence resonance energy transfer (FRET) experiments could be designed such that small molecules could be screened for inhibition or induction of FRET between...
Figure 5
Podophyllotoxin-induced apoptosis of mink lung cells. (a) Photolysis releases podophyllotoxin from beads. (b) Top panel, non-photolyzed podophyllotoxin does not induce apoptosis. Lower panel, UV-released podophyllotoxin induces apoptosis only within the droplet that contains a bead (right). (c) Higher magnification view of a photolyzed droplet without (left) and with (right) a bead. In the one with a bead (right) cells display characteristic blebbing, indicative of apoptosis.

Significance
The arrayed nanodroplet technique allows large collections of small molecules prepared by split-pool synthesis to be assayed with mammalian cells without complications arising from diffusion. Droplets of approximately 50-150 nl result in a significant miniaturization of assays, making this method of detecting ligands with biological activity an efficient means of screening. In a similar way to screening thousands of colonies on an agar plate, it is now possible to screen many thousands of droplets on a molded plastic device. The main features of this method include formation of uniformly sized droplets and a mild wetting-dewetting procedure that enables mammalian tissue culture to be used in assays. The arrayed nanodroplet format therefore provides a means for the ligand-based approach to become a general one for studying the cellular function of proteins.

Materials and methods
Fabrication of master plates and plastic devices for arrayed wells
Master plates were prepared by standard photolithographic techniques. Masks were designed for photolithography with a CAD program (Macromedia Freehand). Using a commercially available high-resolution printer (Herkules PRO, 3386 dpi, Linotype-Hell Company), the designs were transferred to transparencies that in turn served as photomasks for lithography [18]. Depths of the wells were controlled by the type of photoresist used and the rate at which the photoresist was spin-coated. Negative photoresist (SU-8 50, Microlithograph Chemical Corporation)
was spin-coated at 3,000 rpm to produce 40 µm deep wells and at 1,500 rpm to produce 150 µm depth. Plastic devices molded from the masters were produced by casting polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning) prepolymer onto the master. After curing for 4 h at 85°C, the polymer was peeled away from the master. A single master can be used for many cycles (>20) of molding [19,20].

**Distribution of beads in wells**

Tentagel beads were washed once with ethanol, then incubated in 0.01% Triton-X in water for 1 h to dissociate beads and prevent clumping. After incubation with the detergent, the beads were washed twice with water. A 10 mg/ml bead mixture was prepared using 0.006% agarose in water. The surface of the plastic was wetted and dewetted with the bead mixture. A glass coverslip was used to aid in spreading the bead-containing droplets. The excess bead mixture was pipetted off from a corner of the plastic. The beads in wells were visualized using an inverted microscope (Olympus CK2) at 40X magnification. In order to prevent evaporation, a pool of isotonic solution was placed around the PDMS nanodroplet array and the petri dish was quickly sealed with a double layer of parafilm.

**Yeast growth inhibition assay**

A specific ligand immobilized onto beads originally prepared using split-pool synthesis (J. Morken, J. Huang and S.L.S., unpublished results) that was selected from a library of 125,000 compounds [21] was incubated in 0.01% Triton-X in water for 4 h. The beads were washed in YPD complete medium, then diluted with YPD medium to a concentration of 1 mg/ml. Yeast was added to the bead mixture such that each droplet contained approximately 100 yeast cells. Two PDMS plates were placed in separate 10 cm petri dishes and wetted with the bead and yeast mixture for 2 min, then dewetted, with excess liquid pipetted off. YPD medium was added to surround the PDMS plates, and the petri dishes were quickly sealed with parafilm to prevent evaporation. One plate was irradiated under a long wavelength UV lamp at 365 nm (Blak Ray, Model B 100 AP) while sitting in a pool of cool water to prevent heating during the 1 min irradiation period. The other plate was left untreated with no UV irradiation. The plates were incubated in a 30°C incubator for 24 h, then visualized using an inverted microscope (Olympus CK2) at 100X magnification.

Podophyllotoxin-induced apoptosis of mink lung cells plated in microwells

PDMS plates were sterilized by autoclaving. A 0.5 mg/ml solution of fibronectin in PBS was wetted and dewetted onto the PDMS plates to form droplets. The droplets were allowed to evaporate at room temperature for 2 h while the fibronectin deposited onto the wells. The wells were washed three times with PBS buffer and then allowed to dry. Confluent mink lung cells (MvLLu) from a tissue culture flask were washed in YPD complete medium, then diluted with YPD medium to a concentration of 10 000 cells/ml. MvLLu cell mixture was pipetted over the entire surface of the PDMS plates and cells were allowed to settle and attach to the fibronectin-coated wells overnight. The excess media was dewetted and a 2 mg/ml mixture of podophyllotoxin-immobilized beads in media was wetted and dewetted over the PDMS surface to form droplets with and without beads. Media was pipetted to surround the PDMS plates that were then placed in 10 cm petri dishes and the dishes were quickly sealed with parafilm to prevent evaporation. One plate was irradiated for 1 min at 365 nm under the same conditions as the aforementioned yeast inhibition assay. The other plate was left untreated, without UV irradiation. Plates were incubated in a 37°C CO2 incubator for 4 h. Cells were visualized using an inverted microscope (Olympus CK2).

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