Small molecule-dependent genetic selection in stochastic nanodroplets as a means of detecting protein–ligand interactions on a large scale

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Background: Understanding the cellular role of a protein often requires a means of altering its function, most commonly by mutating the gene encoding the protein. Alternatively, protein function can be altered directly using a small molecule that binds to the protein, but no general method exists for the systematic discovery of small molecule ligands. Split-pool synthesis provides a means of synthesizing vast numbers of small molecules. Synthetic chemists will soon be able to synthesize natural product-like substances by this method, so compatible screening methods that detect the activity of minute quantities of molecules among many inactive ones will be in demand.

Results: We describe two advances towards achieving the above goals. First, a technique is described that uses a simple spray gun to create 5000–8000 droplets randomly, each having a volume of 50–200 nanoliters. The individual 'nanodroplets' contain a controlled number of cells and many also contain individual synthesis beads. As small molecules can be photochemically released from the beads in a time-dependent manner, the concentration of ligands that the cells are exposed to can be controlled. The spatial segregation of nanodroplets prevents the mixing of compounds from other beads so the effects of each molecule can be assayed individually. Second, a small molecule-dependent genetic selection involving engineered budding yeast cells was used to detect intracellular protein–ligand interactions in nanodroplets.

Conclusions: The technique described here should facilitate the discovery of new cell-permeable ligands, especially when combined with a positive selection assay that detects intracellular binding of small molecules to proteins. Using 'anchored combinatorial libraries', it may be possible to screen entire libraries of natural product-like molecules against the entire collection of proteins encoded within cDNA libraries in a single experiment.

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Introduction

Determining the cellular function of a protein generally requires a means of altering its function. Most frequently this is done indirectly by mutating the gene encoding the protein. The majority of mutations used to study protein function inactivate the encoded protein. The most common of these are deletion mutations, which are widely used throughout genetic research. Also useful are conditional mutations, where the inactivating effect is only observed under 'non-permissive' conditions controlled by the experimenter. An example is a temperature-sensitive folding mutation. Shifting to a non-permissive temperature prevents newly synthesized polypeptide chains from folding, and therefore from acquiring their cellular function. Mutations that activate the function of encoded proteins are also known. The most notable of these are oncogenic mutations that cause constitutive activity in signaling proteins normally under cell cycle regulation. Conditionally activating mutations are extremely rare.

Protein function can also be elucidated by directly altering the function of a protein through the binding of a small-molecule ligand. Cell-permeable ligands are particularly valuable in this respect, since they allow the study of proteins within their cellular environment. Like genetic mutations, ligands can either inactivate or activate the function of the proteins to which they bind. Importantly, they do so in a conditional way — alteration of function requires that the molecule be included in the experiment. Since small molecules that inactivate function have been used in an analogous fashion to temperature-sensitive folding mutations, the specificity of the two techniques is of interest.

Recent results examining the effects of small molecules and temperature shifts on a complete set of transcripts suggest exquisite specificity in the former, but not latter case ([1]; S. Friend, personal communication). This specificity is also seen with at least one class of molecules that activate function. These molecules have dimeric structures capable of binding two proteins simultaneously ('dimerizers') [2]. By simply causing two proteins to have a proximal relationship in the cell (provided they also have a suitable relative orientation), activation of one of the proteins can ensue. This is due to the increased rate of chemistry (e.g. phosphorylation, proteolysis) that can occur between appropriately selected pairs of proteins when their effective molarity is enhanced by ligand-dependent proximity [3].

The past ten years have provided numerous illustrations of the equivalency of ligands and mutations in the study of cellular protein function [4,5]. Thus far, with only a few exceptions, the ligands used have been either natural products or their synthetic variants. To extend the ligand-based approach, powerful methods of ligand discovery are being developed. The principles defined by geneticists to identify mutations that illuminate protein function are proving to be of value in the search for new ligands with similar properties. Geneticists generate large numbers of mutations, choose from the myriad of methods to prepare the library of mutations and select the desired mutations through the use of an effective screen. In the future, 'chemical geneticists' might synthesize large numbers of small molecules, for example, by using split-pool synthesis [6,7] while choosing from the numerous methods available to synthesize natural product-like molecules, and then select the desired ligands through the use of screens compatible with the split-and-pool method of small molecule generation [8,9].

Although only certain aspects of this approach to ligand discovery have yet been tested in the laboratory, it has been used widely in nature to produce natural product ligands. For example, bacterial geneticists have uncovered the global outline of polyketide synthesis, leading to members of this class that include rapamycin and FK506 [10,11]. These molecules are synthesized by an iterative sequence involving a Claisen condensation, ketone reduction, dehydration and enone reduction. The polyketide synthases containing the enzyme modules that perform these functions are encoded in single bacterial operons. These modules appear to have been shuffled throughout evolution by genetic recombination, which split-pool synthesis emulates. Other types of gene modifications, for example the mutations in the ketoreductase modules, further enhance the structural complexity of the natural polyketide library. Finally, the process of natural selection leads to the existing members of this family of ligands. Their frequent use in present day cell biological studies stems from their selection, over perhaps a billion years, as protein ligands.

In this paper we describe two advances that provide the means to detect small molecule–protein interactions within yeast cells on a large scale. The technique involves the stochastic generation of large numbers of tiny cell cultures named nanodroplets. It is compatible with split-pool synthesis and can be used to identify molecules that bind to a single protein target, to a collection of targets or possibly even to the entire collection of proteins encoded within a cDNA library. In the accompanying paper [12] we describe a complementary technique for generating arrayed nanodroplets and demonstrate their use to detect small molecule–protein interactions within mammalian cells.

Results

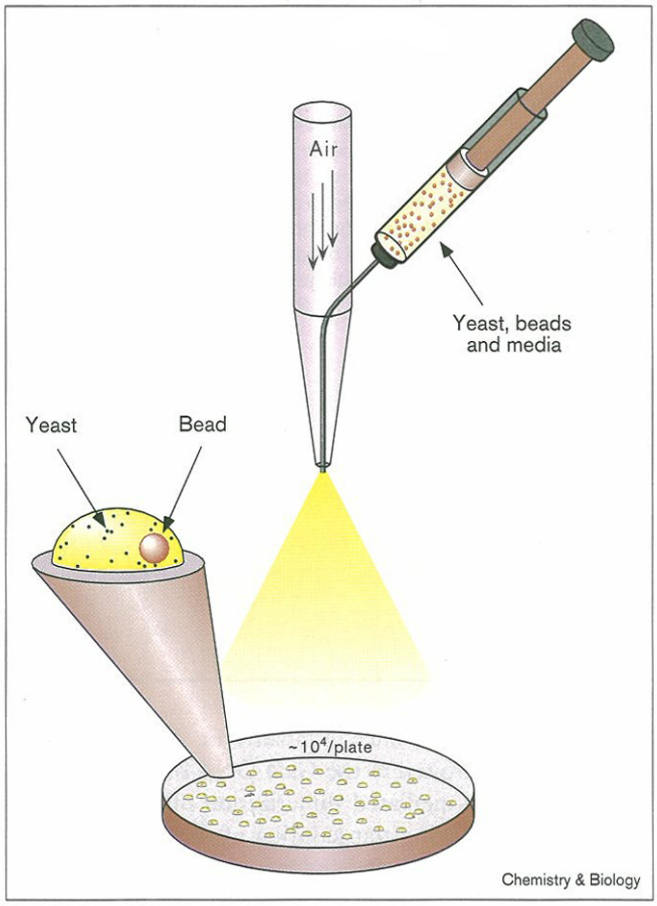
Generation of stochastic nanodroplets

To generate droplets of similar size, with a high proportion of droplets containing a single bead, we found that it was important to create a very fine mist of media and beads while spraying. The application of multiple layers of the mist results in the formation of nanodroplets having a narrow range of volumes. The average size of the droplets is controlled by the amount of media or liquid sprayed, with the spraying of more media leading to fewer droplets of larger volume.

Several factors including construction of the sprayer, media viscosity, bead preparation and air pressure affect the fineness of the mist and therefore droplet formation. The sprayer is built from an 18-gauge, 1.5-inch needle and a 1 ml disposable plastic pipette tip (Figure 1). The most important consideration in construction of the sprayer is that the needle tip be positioned exactly in the center of the pipette tip opening. If the needle tip touches the pipette tip opening while spraying, the medium splatters onto the surface, giving less than optimal results. The medium is prepared as a low percentage agar solution, which serves to increase the viscosity of the liquid, thereby keeping the beads evenly dispersed. Without agar, the beads tend to settle and aggregate while being sprayed, resulting in a higher percentage of droplets containing multiple beads. Prior to suspending the beads in media, they should not be aggregated, which is accomplished by sonicating them in dimethylformamide (DMF), then methanol, and finally washing them extensively with water to remove any residual solvent. Finally, the beads and media are mixed with cells and the resulting suspension is sprayed by slowly injecting it into a stream of air at a pressure of 1–2 pounds per square inch. If the air pressure is too low or the injection rate is too high, spattering, rather than a fine mist, results.

As might be expected, the percentage of droplets containing beads, and therefore the number of beads that can be screened in a given area, is a function of the density of beads in the media prior to spraying. When a 20 mg/ml mixture of beads is sprayed, we get an average of 19 bead-containing droplets per square centimeter. Thus, it is possi-

Figure 1



Formation of nanodroplets by spraying. A mixture of beads evenly dispersed in medium containing yeast is slowly injected into a stream of air forming a fine mist. When layered on to a surface such as a Petri dish this forms into nanodroplets. The average volume of the droplets is controlled by the amount of liquid applied to the surface. For a droplet volume of 50–200 nl it is possible to deposit 5000–8000 droplets in the area of a Petri dish (80 cm^2). The fraction of droplets containing beads depends on the density of beads in the medium prior to spraying. When a mixture of 80 μm Tentagel beads and medium are sprayed at a density of 14,000 beads/ml, approximately 10% of the droplets contain beads. This results in 1000 bead-containing droplets per Petri dish. Of the bead-containing droplets we find that 88% contain a single bead, 10% contain two beads, 1.3% contain three beads, and 0.7% contain four beads.

ble to conduct approximately 2000 experiments in the area of a 96 well plate. Of those droplets, we find that 88% contain one bead per droplet, 10% contain two beads per droplet, 1.3% contain three beads per droplet and 0.7% contain four or more beads per droplet. Of the total number of droplets, approximately 10% contain beads. By increasing the concentration of beads it should be possible conduct more experiments per unit area, at a cost of more droplets containing multiple beads.

Because of their small volume, droplets tend to evaporate quickly if left open to the atmosphere. To prevent evaporation of the droplets once they have been sprayed, they are covered, parafilmed, and stored in a humid atmosphere. We use a plastic bag lined with a moist paper towel to create the moist atmosphere. Using this technique we have stored droplets for up to three weeks in an incubator at 30°C or at room temperature without noticeable evaporation.

Preparation of rapamycin test beads

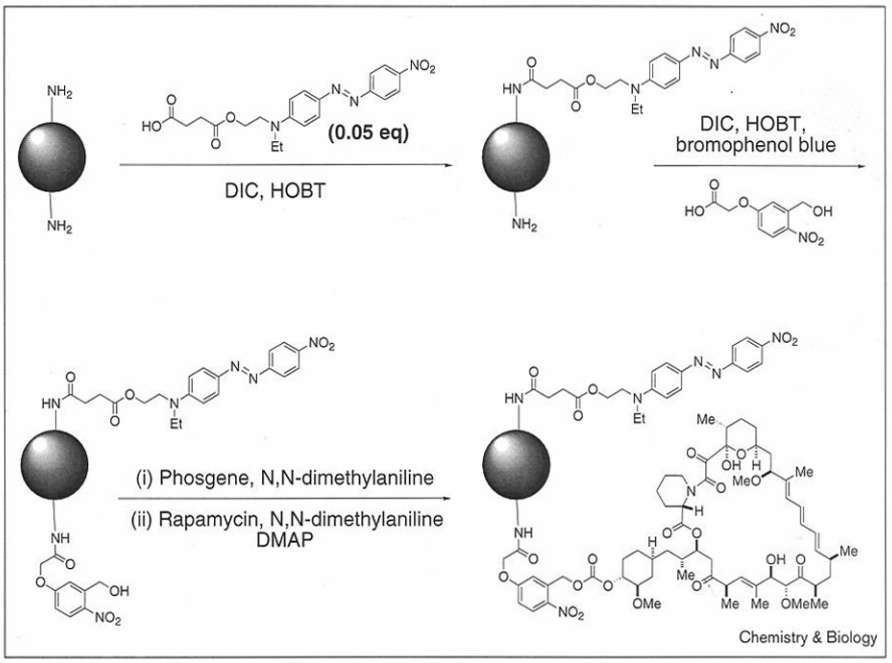
We prepared photocleavable rapamycin beads to investigate yeast growth inhibition and induction in nanodroplets (Figure 2). Rapamycin was attached via a photocleavable linker so that it could be cleaved from resin without addition of external reagents that might interfere with the assays, and so that the amount of rapamycin released could be controlled by the length of irradiation with ultraviolet (UV) light. We chose the 4-alkoxy-2-nitrobenzyl carbonate linker (T.J. Mitchison, personal communication) because it is efficiently cleaved by light, and because it releases rapamycin in an unmodified form). Rapamycin has three hydroxyls through which attachment to the linker could occur. Its cyclohexyl hydroxyl is more reactive towards acylation, but regardless of where attachment occurs, cleavage releases rapamycin.

In order to make the rapamycin beads easily distinguishable from control beads used in our experiments, we labeled the rapamycin beads by chemically attaching a highly colored dye. Starting with TentaGel-NH_2, approximately 5% of the amino groups were acylated with the succinic half ester of Disperse Red. The remaining amino groups were used for attachment of the photocleavable linker. The red resin functionalized with the photolinker was converted to the chloroformate by treatment with phosgene, and then reacted with rapamycin in the presence of 4-dimethylaminopyridine (DMAP) and dimethylaniline. Rapamycin cleaved from the resin was indistinguishable from an authentic sample of rapamycin by chromatography, mass spectrometry and biological assays. The presence of the dye had no noticeable effect on cleavage of rapamycin from the resin.

Growth inhibition assay

In order to test whether we could release a bead-attached compound by photolysis, deliver it into cells, and observe a cellular effect within the small volume of a nanodroplet, we used an experiment that detects binding of rapamycin to its protein targets FKBP12 and TOR1p/TOR2p (the yeast homologs of the mammalian FKBP12-rapamycin-associated protein, FRAP). In wild-type *Saccharomyces cerevisiae* yeast, binding of rapamycin to both FKBP12 and TOR1p/TOR2p simultaneously, results in G1 arrest [13], and as a result, inhibition of growth. In this experiment, red photocleavable rapamycin beads, and colorless control beads containing no compound, were sprayed in YPD media containing 0.063% agar and yeast at a concentration of 10^6 cells per ml (approximately 100 cells per 100 nl droplet). The resulting beads and droplets were irradiated

Figure 2



Synthesis of red photocleaveable rapamycin beads. Tentagel resin is partially acylated (~5%) with the succinate half ester of Disperse Red. The remaining amine functionality is acylated with the nitrobenzylalcohol photocleavable linker, then converted to a chloroformate by reaction with phosgene, and finally linked to rapamycin using catalytic 4-dimethylaminopyridine (DMAP). The rapamycin beads are made red to make them visually distinguishable from colorless control beads. DIC, 1,3-diisopropylcarbodiimide.

with long wavelength UV light (365 nm) for times between 15 s and 5 min, then incubated at 30°C for 24 h to allow the yeast to grow (Figure 3). We found that rapamycin was indeed released from the beads, and that it did inhibit yeast growth depending on the length of irradiation. Irradiation for 15 s released enough rapamycin to inhibit growth in approximately 75% of the droplets containing rapamycin beads. With photolysis times greater than or equal to 30 s, growth was completely inhibited. Growth was normal in droplets containing no beads, or in droplets containing only control beads. Growth was also normal if photolysis was omitted, indicating that rapamycin was released by photolysis, and not by some other means (ie., hydrolysis).

Growth induction assay

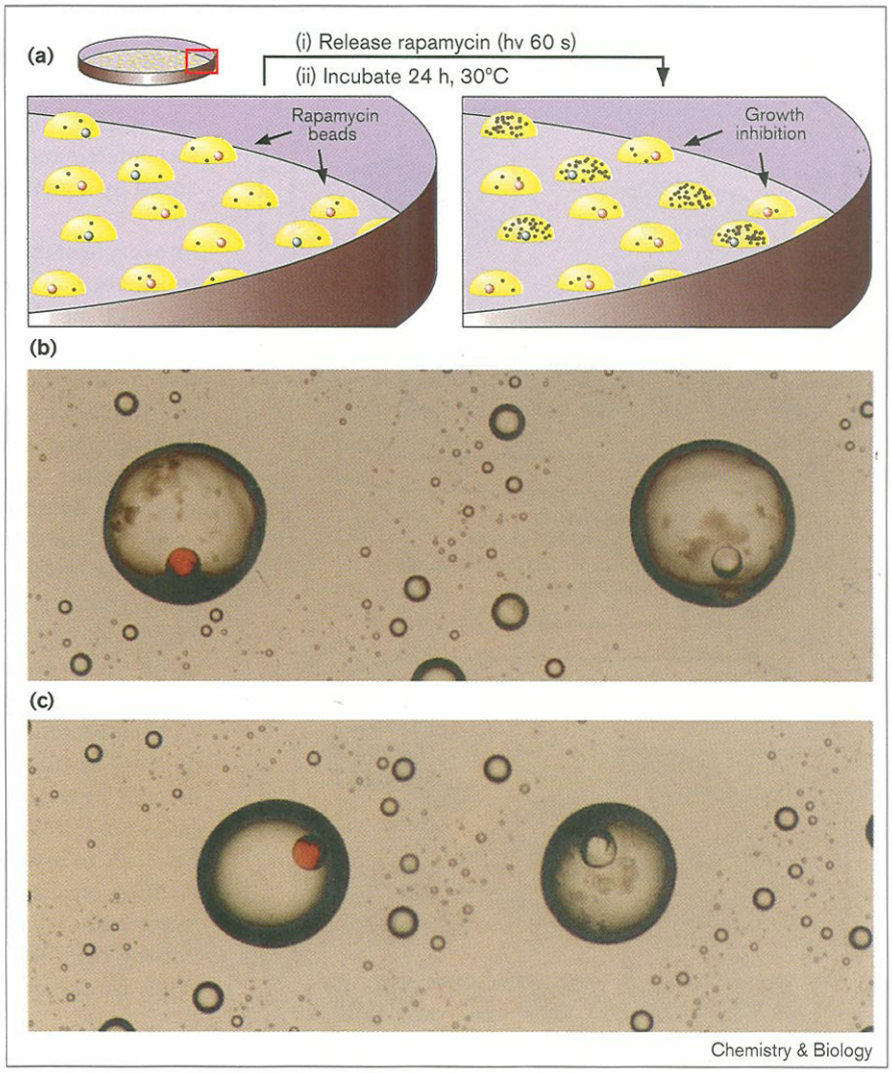
To explore the possibility of using growth, rather than inhibition of growth as a readout for small-molecule binding to a protein target in nanodroplets, we used rapamycin to dimerize FKBP12 and FRB. FRB is a small, 100 amino-acid domain of FRAP that binds to the complex of rapamycin and FKBP12 [14]. In order to conduct this experiment in yeast, in which rapamycin is normally toxic, it was necessary to use a rapamycin-resistant strain. The strain Y153 was engineered to be rapamycin resistant by deleting endogenous FKBP12 and mutating Ser1972→Ile in the *TOR1* gene [15]. This mutation prevents binding of rapamycin to the FRB domain of TOR1 by creating a 'bump' on the FRB surface (Figure 4). The resulting strain, YDF6, was transformed with two fusion proteins: the GAL4 DNA-binding domain (GAL4BD) fused to FKBP12, and the FRB domain fused to the Gal4 activation domain (GAL4AD). In the presence of rapamycin, the Gal4 activation domain is recruited to the Gal4 DNA-binding domain by the rapamycin-mediated dimerization of FKBP12 and FRB, thereby initiating transcription of the *HIS3* reporter gene. As this strain lacks the endogenous *HIS3* gene, these yeast will only grow if histidine is supplied externally or, in the case where histidine is not supplied externally, if rapamycin is present. Rapamycin induces the production of His3p protein in the cells, and thus allows them to grow in histidine dropout media.

These yeast were grown in selective media to mid-log phase, pelleted, resuspended at a concentration of 100 cells per 100 nl droplet in media lacking histidine, mixed with rapamycin beads and control beads, and then sprayed. The resulting droplets were photolyzed for 60 s and incubated at 30°C for up to 7 days. Growth became apparent in some of the droplets in as little 3 to 4 days. After 6–7 days, growth was apparent in >90% of droplets containing the rapamycin-bearing beads. Importantly, growth was never observed in droplets containing control beads or no beads at all. We found that to get a high percentage of growth in droplets containing the rapamycin-bearing beads, consistently, it was important to wash the beads thoroughly to remove residual solvent, and to use mid-log phase yeast.

Discussion

We have developed a simple technique for performing cell culture assays with small molecules, including molecules produced by split-pool synthesis. A spray gun is used to disperse a mixture of cells, defined media and beads containing small molecules prepared by synthesis (Figure 1).

Figure 3

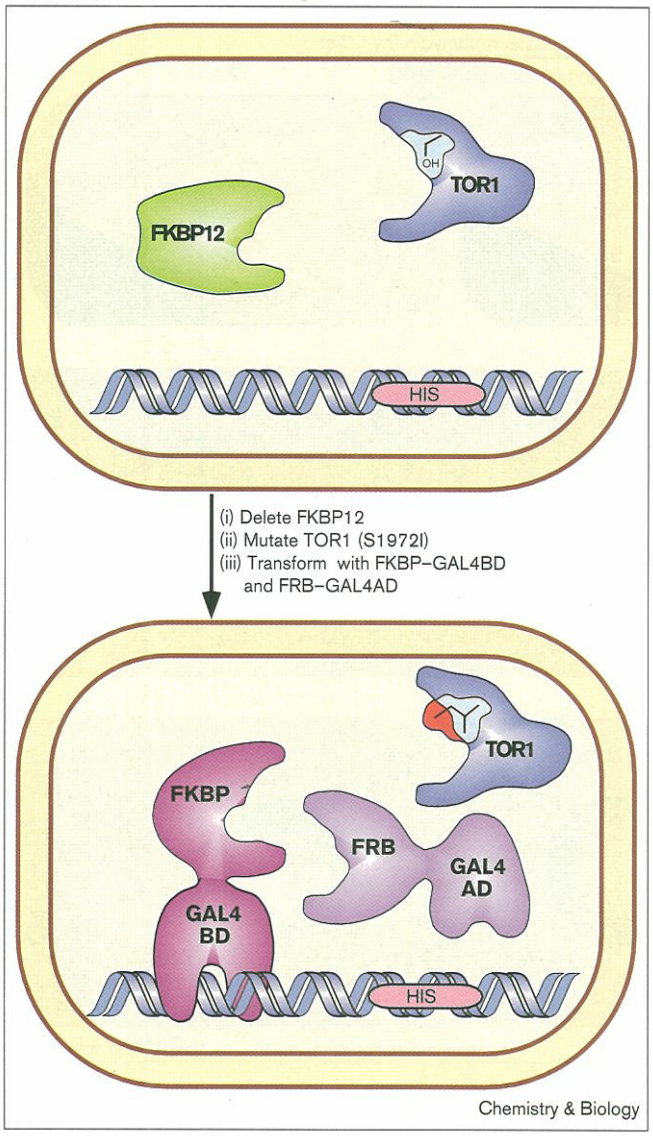


Growth inhibition of wild-type yeast by rapamycin beads in nanodroplets. **(a)** Diagram depicting a growth inhibition experiment with red photocleaveable rapamycin beads and colorless unfunctionalized control beads. Photolysis releases rapamycin from the red beads, inhibiting yeast growth in those droplets. Normal growth is observed in droplets containing the control beads or no beads at all. **(b)** Droplets that have not been photolyzed. Yeast growth is normal when photolysis is omitted, indicating that rapamycin release is light-dependent. The dark patches on the droplets are microcolonies of yeast. **(c)** Droplets that were photolyzed for 60 s then incubated for 24 h. Growth has been inhibited in the rapamycin bead droplet (left), as indicated by the lack of microcolonies. In the adjacent control bead (right), droplet growth is normal, indicating that there is no diffusion of rapamycin to nearby droplets.

The spraying technique, and the wetting–dewetting technique described in the accompanying paper [12], produce nanodroplets that are too small in volume and too large in number to conveniently add subsequent reagents. We have therefore developed several assays that provide optical detection of small molecule–protein binding events, without the addition of external reagents. Two such assays, one involving negative and the other positive growth selection, are reported here in the form of two proof-of-concept experiments. Beads containing the small molecule rapamycin attached via a photolabile linker and encoded with a readily observable red dye were prepared for this purpose (Figure 2).

The first experiment detects the intracellular binding of rapamycin to its protein targets FKBP12 and TOR1p/TOR2p (the yeast homologs of the mammalian FRAP protein). In general, a binding event in this assay results in the inability of cells to undergo cell division and thus the absence of colonies in the nanodroplets. This experiment uses wild-type *S. cerevisiae* cells and can be viewed as a chemical equivalent of the classic genetic screen for temperature-sensitive mutants of genes involved in cell division (cdc screen). In a control experiment, nanodroplets containing beads and cells were not photolyzed; after 24 h, microcolonies of yeast cells were observed in all nanodroplets, including those containing the red rapamycin beads. Thus, budding yeast cells are capable of undergoing repeated rounds of cell division in nanodroplets, and rapamycin has no effect on cell division while covalently attached to a bead. In contrast, brief, long wavelength UV irradiation following spraying releases a small fraction of rapamycin from the red beads, preventing proliferation of yeast cells in all nanodroplets containing a red bead. We estimate that a 60 s irradiation releases around 0.01% of the rapamycin, resulting in around 0.1 μM concentration. Importantly, the rapamycin-induced cell cycle arrest is restricted to the nanodroplet containing the red bead in every instance. Thus,

Figure 4



Engineering of rapamycin-inducible yeast. Endogenous FKBP12 was deleted from the starting strain (Y153) to minimize competitive binding to rapamycin. The Ser1972→Ile mutation in TOR1 abolishes rapamycin binding, thereby rendering the cells resistant to the normally toxic rapamycin. Transformation of the resulting strain (YDF6) with two fusion proteins, FKBP–GAL4BD and FRB–GAL4AD, allows rapamycin-inducible transcription of the *HIS3* gene, which in turn allows selectable growth in medium lacking histidine.

diffusion of the released rapamycin molecule to neighboring droplets is not observed (Figure 3).

The second experiment detects the intracellular binding of rapamycin to FKBP12 and FRAP. In general, a binding event in this assay results in a gain in the ability of the engineered cells to undergo cell division, which is indicated by the presence of colonies in the nanodroplets. This 'three-hybrid' assay [16,17] uses budding yeast cells deleted in an essential gene and transformed with an inducible version of that gene. Its induction is dependent upon a small molecule binding to a protein. The system is designed to couple a binding event with the recruitment of a transcription activation domain to the promoter of the essential gene. For the test system, the non-essential endogenous *FKBP12* gene was deleted and a rapamycin-resistant *TOR* gene was introduced by homologous recombination (Figure 4).

In every instance, we observed the photolysis-dependent appearance of colonies only in nanodroplets containing the red beads, consistent with the design concept (Figure 5a,b). This experiment demonstrates that we are able to link the binding of a small molecule to a protein with the ability of cells to grow colonies in nanodroplets. Although the first test system involved a natural heterodimerizer, rapamycin, we have previously shown that synthetic dumbbell-shaped heterodimerizers can also be used to detect binding events within cells [16]. In that work, the binding of one end of a dumbbell-shaped molecule, named FK–CsA (a covalently linked dimer of FK506 and cyclosporin A), could be detected in mammalian cells by two different techniques. In one technique, related to that reported here, binding of FK–CsA to a fusion protein composed of cyclophilin and an activation domain resulted in the recruitment of the activation domain to the promoter of a target gene, inducing transcription of that gene. In a second technique, binding of FK–CsA to a fusion protein composed of FKBP12 and a nuclear localization sequence (NLS) resulted in the recruitment of the NLS to a fluorescent target protein, causing the fluorescent protein, previously located in the cytoplasm, to translocate to the nucleus of the cell.

In an extension of these dimerization experiments, we are currently using split-pool synthesis to create 'anchored combinatorial libraries' consisting of large numbers of combinatorial 'display' molecules, all covalently linked to a constant 'anchor' molecule (Figure 5c). The function of the anchor molecule is to allow a combinatorial molecule to be displayed from an anchor receptor (AR) that binds the anchor molecule with high affinity. We are using this strategy to display a combinatorial library from an anchor molecule receptor attached to a DNA-binding domain (DBD), and to screen for binding to proteins fused to an activation domain. By analogy with the rapamycin-induced growth experiments presented above, binding of the combinatorial molecule to the target should permit growth in defined media. Using this yeast assay in conjunction with nanodroplets, it should be possible screen a library of compounds against a library of targets in a single experiment if a cDNA library of potential targets is attached to the activation domain. Towards this goal, we are currently systematically investigating the relationship of small molecule–protein affinity to the ability of a dimerizer to induce growth in this assay (A.B., unpublished

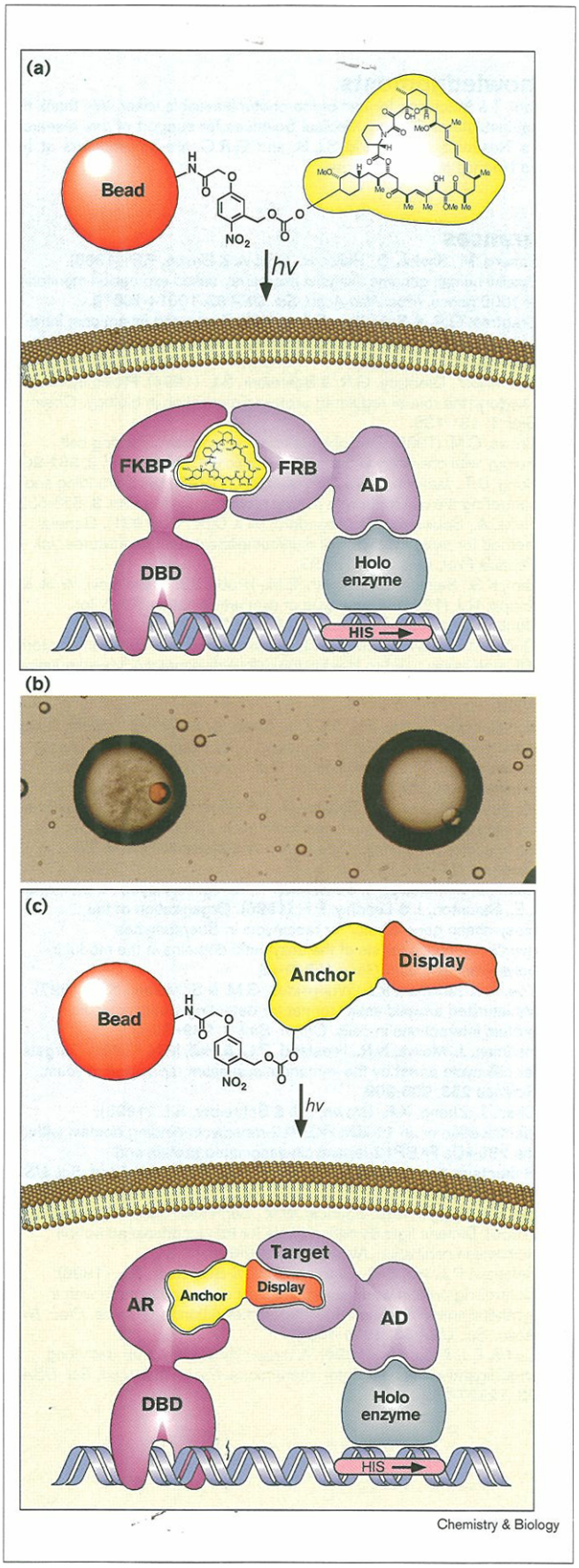


Figure 5

Detection of protein–ligand interactions in nanodroplets. **(a)** Rapamycin-induced yeast growth in engineered yeast. Release of rapamycin from its bead by photolysis results in dimerization of FKBP–BD and FRB–AD (FRB with an activation domain), causing transcription of the downstream *HIS3* reporter gene, enabling the yeast to grow in droplets lacking histidine. **(b)** Rapamycin-induced yeast growth in droplets. The droplet containing the red rapamycin bead (left) shows microcolony formation after 60 s irradiation and incubation for 6 days, whereas the droplet containing a colorless 'blank' control bead (right) showed no detectable growth under the same conditions. **(c)** Screening a combinatorial library in yeast using molecules containing a constant 'anchor' molecule attached to a combinatorial 'display' molecule (see text).

results). A more rapid alternative to growth as a readout is the ligand-dependent fluorescent protein-translocation assay described above, which should be well suited for screening such anchored combinatorial libraries in mammalian cells. We are also adapting split-pool syntheses of natural product-like substances to yield large collections of molecules having anchor and variable ends.

Significance

Cell-permeable, small-molecule ligands provide a means of conditionally altering protein function. As a result, they can be used to dissect the function of proteins, in analogy to the use of conditional mutations. One potential source of such compounds is combinatorial chemistry. In particular, split-and-pool synthesis can provide vast collections of compounds, in minute quantities, attached to the synthesis resin. The continued development of this field promises to deliver collections of compounds whose properties will increasingly mimic those of natural products. We have developed screening in nanodroplets as an efficient means of evaluating such collections of compounds in cell-based assays. This approach was demonstrated in two experiments using rapamycin, the first a negative selection and the second a positive selection. In the first experiment, inhibition of TOR1p/TOR2p by rapamycin–FKBP12 caused G1 cell-cycle arrest in wild-type *S. cerevisiae*, resulting in lack of growth. In the second experiment, dimerization of FKBP12 and FRB by rapamycin was detected in droplets with growth as the readout. Rapamycin-induced dimerization of these proteins fused to a DNA-binding domain and an activation domain, respectively, triggered transcription of a downstream *HIS3* reporter gene, allowing selective growth in droplets lacking histidine. In this work, we have demonstrated growth and lack of growth as readouts of small-molecule binding; however, it should be possible to use many other types of visual readouts, such as changes in cell morphology, secretion of reporter enzymes or translocation of fluorescent proteins. A particularly exciting application will be screening libraries of compounds against collections of receptors simultaneously in yeast cells deposited in nanodroplets.

Materials and methods

Sprayer construction

The sprayer was constructed from an 18-gauge, 1.5-inch needle (Becton Dickinson, #305196), and a disposable plastic 1 ml pipette tip (Biomar, #TN1300RS). The point of the pipette tip was trimmed back exactly 5 mm leaving an opening with an inner diameter of 2.0 mm. The tip of the needle was filed blunt, being careful to remove any burs that might obstruct the opening, then bent 45 degrees, 10 mm from the tip. The needle was inserted through a 1 mm hole made 18 mm from the tip of pipette tip until approximately 1 mm of the needle protruded beyond the pipette tip. At this point the bend in the needle rested on the side of the pipette tip wall, but the tip was positioned exactly in the center of pipette tip opening, not touching the sides. It was sometimes necessary to bend the needle slightly one way or the other to center it precisely. Finally, the needle was held in place with a liberal amount (0.25 ml) of 5 minute epoxy.

Bead–medium mixture preparation

Beads (20 mg, 80 μm TentaGel-S NH_2 resin, 0.25 mmol/g) free of aggregation were suspended in freshly prepared medium containing 0.063% agar (Difco Bacto-Agar). The medium was prepared by dissolving 2.5 mg agar in 4 ml medium, then heated to near boiling until homogeneous. The medium was allowed to cool and become viscous over a 2–3 h period before mixing with the beads. To free the beads of clumping prior to mixing them with media/agar, they were sonicated in DMF (1 ml, 15 min), methanol (1 ml, 15 min), then washed in water (10 × 1 ml, 30 min per wash).

Growth inhibition assay

A mixture of red rapamycin beads (10 mg), colorless control beads (10 mg, 80 μm TentaGel-S NHAc resin, 0.25 mmol/g), YPD media (1.0 ml), and agar (0.063%) prepared as described above, were mixed with freshly cultured wild-type *S. cerevisiae* (diluted to 10^6/ml) and sprayed on Petri dishes (10 cm). The dishes were immediately covered, sealed with parafilm, photolyzed with long wavelength UV light for 15, 30, 60, 120 or 300 s (Black ray UV lamp model B 100 AP), then incubated at 30°C for 24 h.

Growth induction assay

A mixture of red rapamycin beads (10 mg), colorless control beads (10 mg, 80 μm TentaGel-S NHAc resin, 0.25 mmol/g), Leu-, His-, Trp-complete minimal medium (1.0 ml) containing 3-aminotriazole (10 mM), and agar (0.063%) prepared as described above, were mixed with freshly cultured YDF6 yeast (spun-down and resuspended in the triple dropout media/agar mixture at 10^6/ ml) and sprayed on Petri dishes (10 cm). The dishes were immediately covered, sealed with parafilm, photolyzed with long wavelength UV light for 60 s, then incubated at 30°C for 6 days (in a plastic bag containing several wet paper towels to minimize evaporation).

Rapamycin bead preparation

Amino resin (400 mg, 0.10 mmol, 0.25 mmol/g; TentaGel S NH_2; RAPP polymer, #S30902) was mixed with Disperse Red 1 succinic half ester (2.1 mg, 0.005 mmol), DMF (1.5 ml), CH_2Cl_2 (1.5 ml), 1-hydroxybenzotriazole (6.8 mg, 0.05 mmol), and 1,3-diisopropylcarbodiimide (7.8 μl, 0.05 mmol), then agitated at room temperature for 2 h. The resulting light red resin was washed with DMF (5 × 5 ml), and CH_2Cl_2 (5 × 5 ml). A portion of the red resin (50 mg, 0.0125 mmol) was mixed with the photolinker (8.5 mg, 0.0375 mmol), 1-hydroxybenzotriazole (5.1 mg, 0.0375 mmol), DMF (0.25 ml), CH_2Cl_2 (0.25 ml), and 1,3-diisopropylcarbodiimide (5.9 μl, 0.0375 mmol), agitated in the absence of light for 30 min, at room temperature, then washed with DMF (5 × 5 ml), and CH_2Cl_2 (5 × 5 ml). The Kaiser ninhydrin test showed no free amino groups. The photocleaveable resin (25 mg, 0.00625 mmol) was mixed with phosgene in toluene (0.5 ml, 20% by weight) and *N,N*-dimethylaniline (7.92 μl, 0.0625 mmol) for 1.5 h at room temperature in the absence of light, then washed with dry methylene chloride (10 ml), under a nitrogen atmosphere. To a portion of the resulting resin (20 mg, 0.005 mmol) was added rapamycin (13.7 mg, 0.015 mmol), *N*-methylmorpholine (3.1 μl, 0.03 mmol), *N,N*-dimethyaminopyridine (0.25 mg, 0.002 mmol), and CH_2Cl_2 (200 μl, dry). The reaction mixture was agitated overnight in the absence of light, then washed with DMF (5 × 2 ml) and methylene chloride (5 × 2 ml), and dried under high vacuum.

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