Frontiers in chemical genetics

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New methods enable the identification of compounds that both induce a specific cellular state and lead to identification of proteins that regulate that state. Together, developments in three critical areas: chemical diversity, phenotype-based screening and target identification, enable the systematic application of this chemical genetic approach to almost any biological problem or disease process.

Classic model organism genetics has been a powerful tool for biologists; it has revealed the molecular basis for such diverse processes as cell division in *Saccharomyces cerevisiae*, programmed cell death in *Caenorhabditis elegans,* and embryonic development in *Drosophila melanogaster*[1–3]. This forward genetic approach uses the following methods to identify genes that regulate a biological process of interest: (1) random mutagenesis of cells or organisms; (2) phenotype-based screening of the cells or organisms; and (3) gene identification by mapping of the selected mutations.

The converse procedure, reverse genetics, has been immensely useful in creating models of human disease in other organisms, especially in mice using homologous recombination (knockout) technology[4]. Reverse genetics requires: first, the selection of a gene of interest; second, the creation of an organism or cell with a mutated version of this gene; and third, a broad search for phenotypic differences between the wild-type and mutant organisms or cells, to identify a biological function for a gene of interest.

Together, forward and reverse genetic approaches constitute a powerful set of tools for dissecting and understanding biological systems. However, despite their enormous usefulness, both approaches have limitations: (1) it is difficult to perform forward genetic analyses in mammalian systems because of the large size of mammalian genomes, their diploid nature and the slow rate of mammalian reproduction; (2) genetic mutations are usually constitutive – they cannot easily be turned on and off, particularly in mammals.

Given that the application of forward genetic screens to mammalian biology and disease would be enormously useful, great efforts have been made to overcome the difficulties of such analyses. Large-scale mutagenesis and screening have been undertaken in both zebrafish and mice[5–8], yielding preliminary results that indicate such monumental efforts might be worthwhile[9,10].

Nevertheless, the practical limitations of performing genetic screens in mammalian systems are illustrated using studies of the molecular basis of human genetic disorders, such as Huntington's disease and Parkinson's disease, which are both fatal neurodegenerative disorders. Although the process took many years, a forward genetic approach (positional cloning) in humans has led to identification of the genetic defects that cause these diseases[11,12]. Even though the mutations that initiate the diseases have been discovered, the downstream molecular events still remain obscure. A forward genetic screen for suppressors of disease pathology would be a powerful method for studying these downstream events, but such screens cannot be performed easily in mammalian systems. Recently, models for Huntington's and Parkinson's diseases have been created in *Drosophila*, allowing a genetic approach towards understanding these disease mechanisms[13–15]. The ability to perform genetic screens in mammalian systems rather than in lower organisms, however, would greatly facilitate studies of the molecular basis of disease processes in humans.

A chemical genetic approach that uses small organic molecules has the potential to overcome the limitations of classic genetic analyses in mammalian systems. In this approach, small molecules are used to directly alter protein function, by binding to their protein targets and by either enhancing or inhibiting the function of the targets[16–18].

Given that these compounds can be added or removed at will, they are, by definition, conditional. They can also act like either gain-of-function or loss-of-function mutations, depending on whether they activate or inhibit the function of their target proteins. Thus, using this strategy, it is possible to identify new reagents that act like conditional mutations, either inducing or suppressing the formation of a specific phenotype of interest, such as the pathology found in cells from patients with Huntington's disease. Furthermore, these reagents can be used to study the mechanistic basis of the phenotype in question, by identifying the protein targets of the reagent[16,18].

An early example of this forward chemical genetic approach was the discovery of the immunosuppressive agent FK506 by researchers at Fujisawa Pharmaceutical Co. Ltd. (Ibaraki, Japan). Before this discovery, the natural product cyclosporin A (CsA) was the standard treatment for preventing organ transplant rejection. CsA operates by inhibiting the production of T-cell-derived soluble cytokines, such as interleukin-2 (IL-2; Ref. 19). Using a phenotype-based screen of extracts that contain natural products, Kino *et al.* tested several fermentation broths for their ability to phenocopy CsA (i.e. block IL-2 production) – thus, they discovered FK506, a natural product that is structurally unrelated to CsA[19,20]. Subsequent studies placed calcineurin, the ultimate molecular target of FK506, on the T-cell-receptor-initiated signaling pathway, thus facilitating the complete elucidation of the molecular events that govern this membrane-to-nuclear signaling pathway[21]. Furthermore, FK506 was developed into a clinically

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useful immunosuppressive agent (tacrolimus), which suggests that this strategy might be of both academic and commercial relevance.

The success realized with FK506 can be extended to any biological process using two important elements: (1) diverse collections of organic compounds that are compatible with high-throughput screening; and (2) miniaturized phenotype-based assays. Advances in both of these fields are discussed in this article.

Sources of small molecules

What types of compounds are optimal for chemical genetic studies? Because there is an inverse correlation between molecular weight and membrane permeability[22], and because membrane permeability is required to modulate the function of intracellular targets, low molecular weight organic species are generally preferred (MW <1500 g mol^{-1}). Because the number of possible carbon-containing molecules with a molecular weight <1500 g mol^{-1} is still astronomically large, the existing synthetic routes to small molecules are the primary limitation, rather than the total universe of theoretical compounds. Classes that are currently synthetically accessible include simple drug-like flat aromatic compounds, complex naturally occurring secondary metabolites (natural products), non-natural foldable polymers and peptides.

Simple compounds

Simple drug-like small molecules are currently available in a wide variety of formats and quantities from commercial suppliers. For the purposes of this review, simple small molecules are those with: (1) a molecular weight <500 g mol^{-1}; and (2) no stereogenic centers (Fig. 1). Screens of simple small molecules have yielded compounds that can activate specific reporter genes[23], arrest cells in mitosis[24], inhibit the function of the tumor suppressor p53 (Ref. 25) and activate a specific subtype of cell-surface receptor[26]. These simple compounds can serve as useful leads for the development of therapeutic agents, and their wide availability makes them an attractive starting point for screening. However, simple compounds might not be able to discriminate between related protein species because the total surface area that is available on simple small molecules for binding to protein surfaces is limited owing to the small size and limited chemical functionality of these compounds.

Complex compounds

Naturally occurring secondary metabolites (natural products) often have a richer density of functionality and stereogenic centers when compared with simple small molecules (Fig. 2). In other words, many natural products have structural complexity, which, for the purposes of this review, is a property assigned to molecules with a molecular weight >500 g mol^{-1} and at least one stereogenic center. Examples of clinically useful complex natural products include cyclosporin, FK506, rapamycin, vinblastine and Taxol (Fig. 2). Although marine sponges, plants and soil have been fruitful sources for the discovery of complex molecules[27], the time and effort that is needed to purify and identify the active species in such extracts ultimately limits their usefulness for chemical genetic analyses.

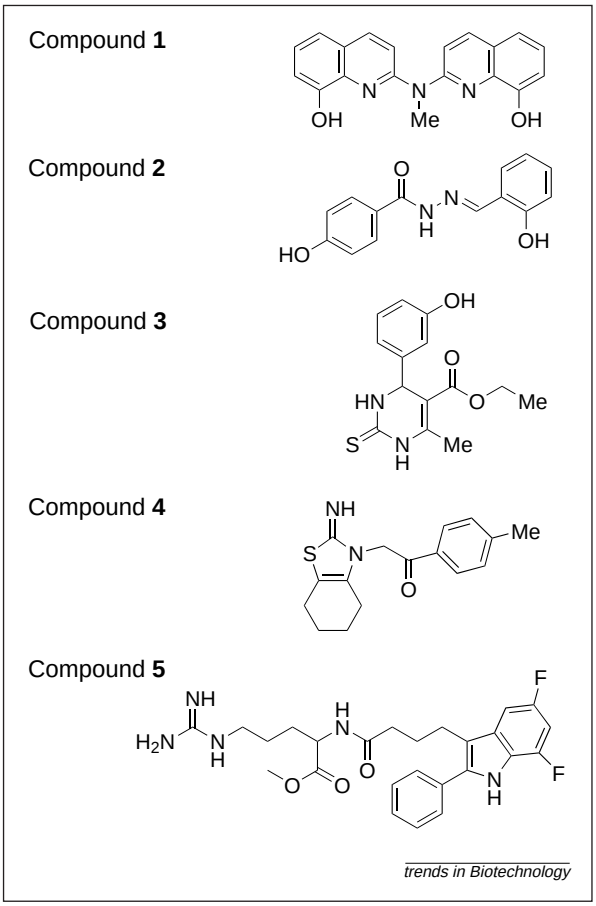

Figure 1

Simple small molecules with biological activity have been identified in chemical genetic screens. Simple small molecules have a molecular weight <500 g mol^{-1} and lack stereogenic centers. Compounds **1** (MW 317 g mol^{-1}) and **2** (MW 256 g mol^{-1}) activate a specific TGF-β-responsive reporter gene[23], compound **3** (MW 292 g mol^{-1}) arrests mammalian cells in mitosis[24], compound **4** (MW 286 g mol^{-1}) inhibits the function of the tumor suppressor p53 (Ref. 25), and compound **5** (MW 485 g mol^{-1}) is a subtype-selective somatostatin receptor agonist[26].

Recently, Handelsman *et al.* described a strategy for purifying natural products from unculturable microorganisms by exploiting techniques of classic bacterial genetics[28–30]. In this approach, the total genomic DNA of microorganisms is isolated directly from soil samples and is digested into large fragments. These DNA fragments are cloned into bacterial artificial chromosomes (BACs), thus creating an expression library with some BAC clones that bear complete operons coding for the biosynthesis of natural products. Subsequently, the BACs are transfected into a culturable host organism, such as *Escherichia coli*, which is grown in isolated wells that each contain a single representative BAC clone. Organic extracts from these wells, containing either a single natural product or a small number of structurally related natural products, are isolated and used in chemical genetic screens. Although this approach is still in the early stages of development, it can theoretically provide an inexhaustible source of structurally novel natural products and should constitute a powerful addition to the arsenal of complex molecules available for screening.

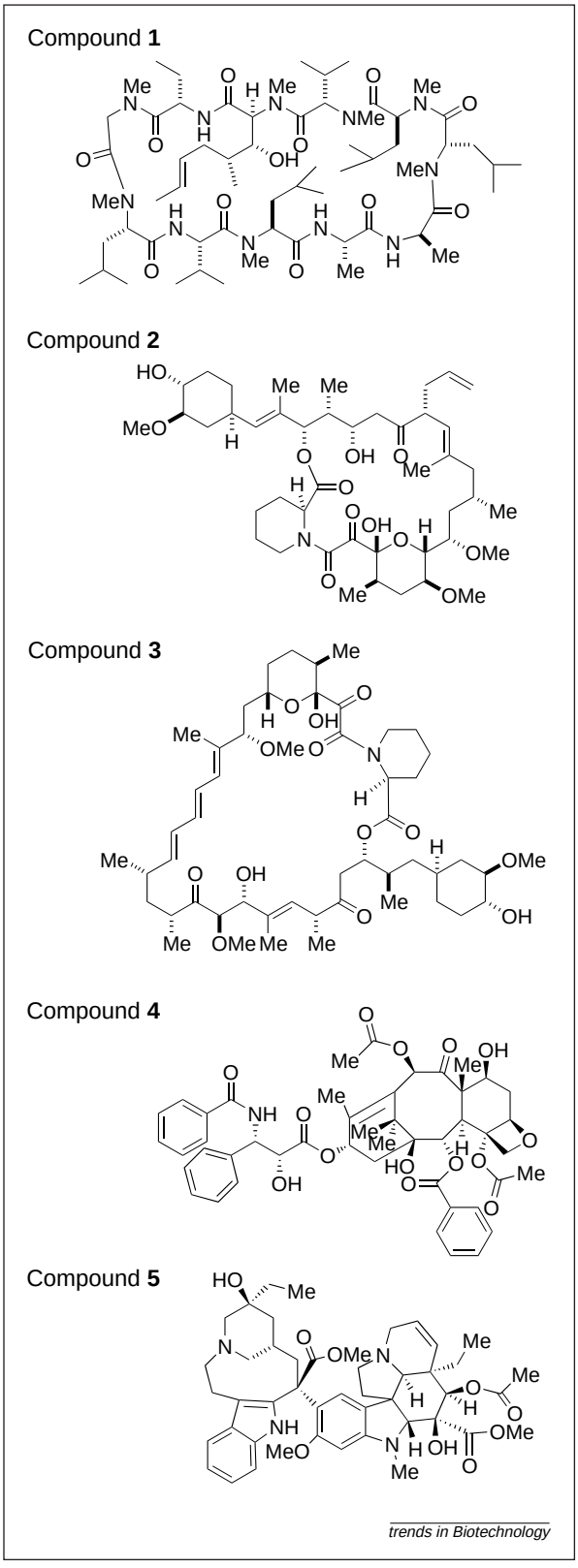

Figure 2

Clinically used natural products that display structural complexity, which is defined as a property of molecules with a molecular weight >500 g mol^{-1} and possessing at least one stereogenic center. Cyclosporin (**1**; MW 1203 g mol^{-1}) and FK506 (**2**; MW 804 g mol^{-1}) are immunosuppressants used to treat organ transplant rejection, rapamycin (**3**; MW 915 g mol^{-1}) is a cell cycle arresting agent being studied for anticancer activity, and Taxol (**4**; MW 854 g mol^{-1}) and vinblastine (**5**; MW 811 g mol^{-1}) are antimitotic agents that are currently used for treating a variety of cancers.

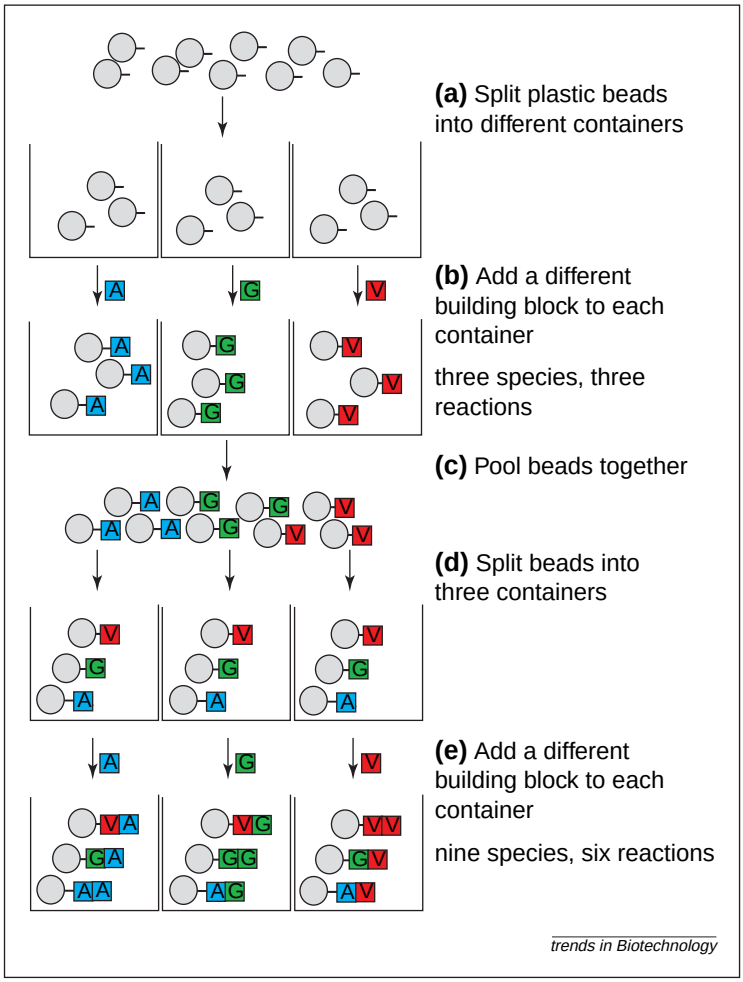

Figure 3

Split-pool synthesis generates many compounds using a small number of synthetic reactions. (**a**) Small (100–500 μm) plastic beads containing a functional 'handle' (e.g. an amine) are divided among several containers (three are shown). (**b**) A different building block, which reacts with the plastic beads, is added to each flask. In the case of peptide synthesis the building blocks are amino acids, such as alanine (A, blue), glycine (G, green) and valine (V, red). (**c**) The beads are removed from their containers and pooled together. (**d**) The beads are divided into three new containers. (**e**) A new building block, which reacts with the existing compounds, is added to each container. The net result is the synthesis of nine different dipeptides (3^2) using six (3+3) synthetic reactions. In the more general case, a synthesis with 'N' number of steps, using 'M' number of containers will yield M^N compounds using M×N synthetic steps.

An alternative to the use of naturally occurring secondary metabolites is the *de novo* synthesis of large numbers of complex natural-product-like small molecules, using automated parallel synthesis or split-pool synthesis[31,32]. Although automated parallel synthesis is commonly referred to as combinatorial chemistry, this designation is inaccurate; the process is more correctly referred to as miniaturized chemistry because it primarily involves traditional chemical synthesis performed in many small wells in parallel. It is only with the split-pool method that the power of combinatorial chemistry is truly realized (Fig. 3). In the split-pool method, each compound is synthesized on a small (100–500 μm) plastic bead, in a process akin to the solid-phase peptide synthesis that was developed by Merrifield[33] (Fig. 3a). By separating and recombining the plastic beads during synthesis, however, it is possible to synthesize a large number of compounds using a small number of

synthetic reactions (Fig. 3; Ref 31). For example, making all possible tetrapeptides (peptides composed of four amino acids) using parallel synthesis would require 1.6×10^5 synthetic reactions ($20 \times 20 \times 20 \times 20$ because there are 20 naturally occurring amino acids). However, using the split-pool method one can synthesize the same set of tetrapeptides using just 80 synthetic reactions ($20 + 20 + 20 + 20$), and at the same time yield a single sequence of tetrapeptide on any given bead (Fig. 3). Recently, the power of this split-pool method was demonstrated with the synthesis of more than two million complex, nonpeptidic small molecules[34,35].

Protein-like foldable organic polymers

All the compounds described previously are rigid small molecules, the conformational properties of which are quite distinct from those of proteins and peptides. Some researchers have attempted to invent new protein-like organic compounds that are capable of forming specific secondary and tertiary structural elements[36]. Given that polymers of amino acids and polymers of nucleic acids are capable of executing highly specific biological functions, it is possible that other compactly folded polymers could exhibit similar properties. In fact, a polymeric backbone composed of specific types of β amino acid adopts a helical secondary structure, similar to that of polymers of α amino acid polymers[36]. Although design of these foldamers is still at an early stage, they might ultimately allow the synthesis of large organic compounds that mimic the diverse functions of proteins, while being impervious to the action of proteases[36,37].

Phenotype-based screens

In addition to libraries of small molecules, forward chemical genetics requires the use of phenotype-based screens with which to assay the small molecules. In this approach, a specific biological process or phenotype is selected for study, a high-throughput assay is designed around that phenotype, and small molecules that enhance or suppress appearance of the selected phenotype are identified.

How does one invent a phenotype-based high-throughput assay? It is helpful to make use of a general model, in which a population of cells exists in one of two states – the wild-type state (state one) or the pathologic state (state two) as shown in Fig. 4. Compounds would be screened and selected on the basis of their ability to interconvert states one and two. The compounds identified in this manner would be powerful tools and potential therapeutic agents because they would be capable of inducing or suppressing formation of a disease state in a rapid and conditional manner. Furthermore, by studying the molecular targets of such active compounds it might be possible to learn about the regulation of a disease state. An example of this general model is the ability of the viral oncogenes *v-ras* and *v-src* to transform fibroblasts from the NIH 3T3 cell line to a characteristic spindly morphology (Fig. 5), and the ability of the natural product depudecin, to revert these transformed cells back to their original flattened morphology[38]. This provides a striking demonstration that a single compound can act to reverse a complex cellular phenotype. Furthermore, the target of depudecin is

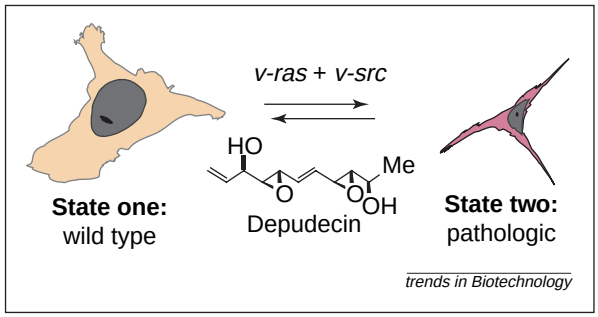

Figure 4

A general model for phenotype-based screens, in which a population of cells exists in one of two states. In this example, the viral oncogenes *v-ras* and *v-src* combine to transform the wild-type state into a pathologic state, as evidenced by a spindly morphology[38]. Furthermore, the natural product depudecin can revert the spindly morphology back to a flattened morphology, which indicates that a single compound can suppress a complex phenotype[38].

a histone deacetylase (HDAC; Ref. 39), which indicates that HDACs might play a crucial role in the process of transformation and therefore might be useful targets for anticancer agents. To rapidly identify modifiers of cellular states, such as depudecin, high-throughput methods are needed that can reliably distinguish between various cellular states.

Markers for phenotype-based assays

Transcription-based markers

High-throughput screens usually employ a marker, which is an easily detectable molecular change representing a broader cellular phenotype. For example, treatment of mink lung cells with transforming growth factor beta (TGF-β) results in a multitude of transcriptional, translational and post-translational changes. Given that a screen for small molecules that induce mink lung cells to enter the TGF-β-treated state could not possibly test for all of these molecular changes, it is necessary to select one specific molecular alteration induced by TGF-β, such as induction of plasminogen activator inhibitor type I (PAI-1) expression, which then serves as a marker for the TGF-β-treated state. Furthermore, because it is technically challenging to detect the presence of PAI-1 itself in a high-throughput manner, a reporter gene (which contains the coding sequence of an easily detectable protein under the control of the genetic promoter of interest) might be used instead. Luciferase, the light-generating enzyme found in fireflies, is preferred for use in reporter gene assays because there are sensitive detection methods for this enzyme and because the luminescent output is not affected by the presence of colored compounds in the chemical library. Therefore, in designing a reporter gene for the TGF-β-treated state, one could put the luciferase coding sequence under the control of the PAI-1 promoter, thus allowing for detection of TGF-β signaling by measuring the amount of light emanating from the cells.

How does one select the promoter element to be used in a reporter gene if no gene is known to be induced in the state of interest? Recent advances in transcription profiling using DNA arrays now allow comparison of global patterns of gene expression in two

distinct cellular states[40]. A gene that is strongly repressed or induced in a disease state can be identified and a reporter gene can subsequently be constructed using the promoter and enhancer elements from this gene. Thus, it should be possible to design high-throughput reporter-gene assays for virtually any cellular state of interest, including any disease state. Alternatively, when high-throughput transcription profiling becomes feasible, the transcription profile could be used as a molecular fingerprint of a specific disease state, and thus allow screens for compounds that revert the pattern of gene expression back to the wild-type pattern.

Post-transcriptional markers

Changes of cellular state often involve a complex array of molecular alterations that are distinct from transcription, such as changes in protein phosphorylation, protein degradation, protein cleavage and subcellular protein localization. Reporter gene assays detect only transcriptional changes and thus they are not adaptable to screens involving these other molecular alterations. Recently, however, a new high-throughput screening method has been developed that allows the detection of virtually any molecular modification in a cell in a high-throughput manner[16]. This technique, known as 'cytoblot', uses a specific primary antibody as a probe to detect the phenotypic change of interest. Cytoblot assays can be used to detect translational and post-translational changes that mark a desired phenotype (Fig. 5) because antibodies can recognize proteins directly, as well as specific protein modifications (such as phosphorylation and acetylation) and conformational changes. In this approach, adherent cells are grown on the bottom of a small well, compounds are added, and ultimately the cells are fixed and permeabilized. An antibody complex is added to the well and binds to the molecular marker of interest within the cells; the presence of this complex is detected in a western-blot-like fashion using enhanced chemiluminescence (Fig. 5; Ref. 16). The final outputs (light emission) of cytoblots and reporter gene assays are identical, which means that existing high-throughput instruments and plates are readily adapted for use with this screening method.

Future phenotype-based screens: morphology and subcellular localization

The most general phenotype-based assay would involve imaging cells or microscopic organisms in an automated fashion and directly observing morphologic changes. Such a method would obviate the need for identifying specific molecular events or pathways that act as surrogate markers for phenotypic changes, and instead would allow direct monitoring of the phenotypes themselves. Pattern recognition algorithms have been developed that enable quantitation of differences between two digitized images, thus allowing the detection of changes in the subcellular localization of a fluorescently labeled protein of interest[41–43]. For example, Murphy *et al.* obtained digitized fluorescence micrographs of immunofluorescently labeled cells, then computed various statistical descriptors of the pattern of pixels appearing within the image[42]. Using these descriptors it was possible to reconstruct a crude approximation of the original image (Fig. 6; Ref. 41). Such a method should be capable of detecting and quantifying changes in the subcellular localization of a specific protein, gross morphological alterations, or many other cellular changes that cannot be detected using other methods[42].

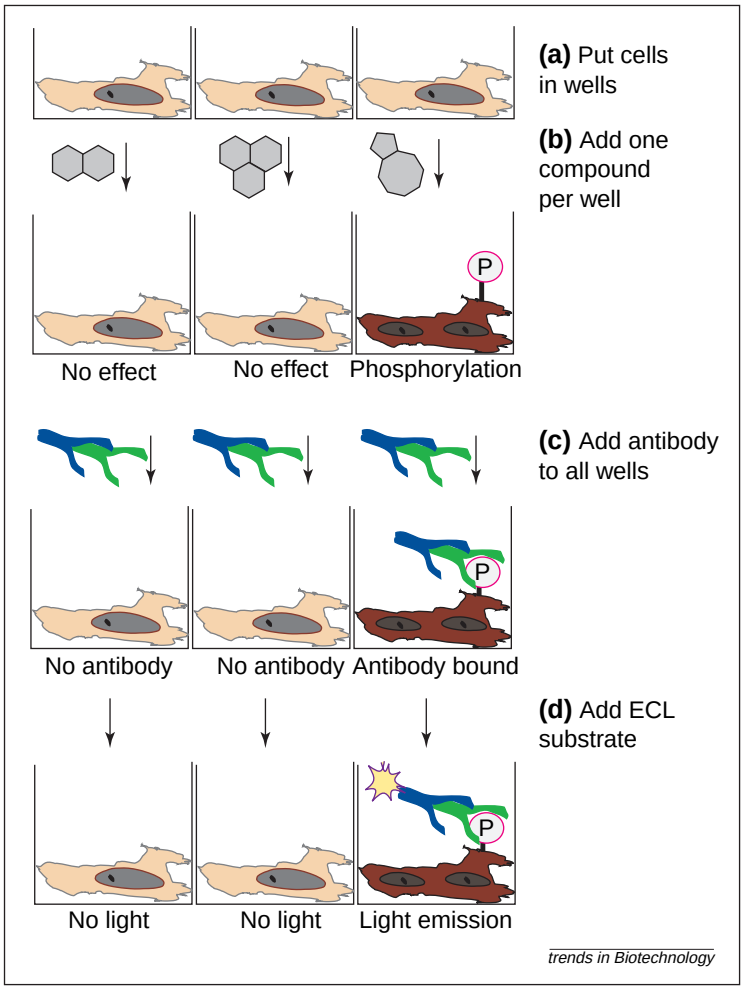

Figure 5

An antibody-based approach to high-throughput screening (cytoblot). (**a,b**) Adherent cells are seeded in small wells and a different compound is added to each well. Some compounds cause the appearance of specific post-translational changes, such as phosphorylation (P). (**c**) An antibody complex is added to the wells and is retained only in those that harbor cells with the molecular alteration recognized by the antibody. (**d**) Finally, the antibody complex is detected using enhanced chemiluminescence (ECL), which is an efficient light-emitting reaction.

Finally, it is often valuable to test in animals, the effects of small molecules discovered in cell-based assays Such studies can reveal the relative importance of a protein target of a small molecule within an organism. For example, a small molecule inhibitor of p53 was used to demonstrate that reversible inhibition of p53 prevents damage to normal tissue from the otherwise lethal genotoxic stress of radiation that is used to treat tumors[25].

Methods of target identification

A major advantage of the chemical genetic approach is that compounds selected in such screens can act both as conditional switches for inducing a cellular state and as probes for identifying protein targets involved in that state. The identification of such targets can shed new light on the mechanisms that regulate the state of interest. Methods for identifying molecular targets of biologically active compounds include classic techniques, such as affinity chromatography and biochemical

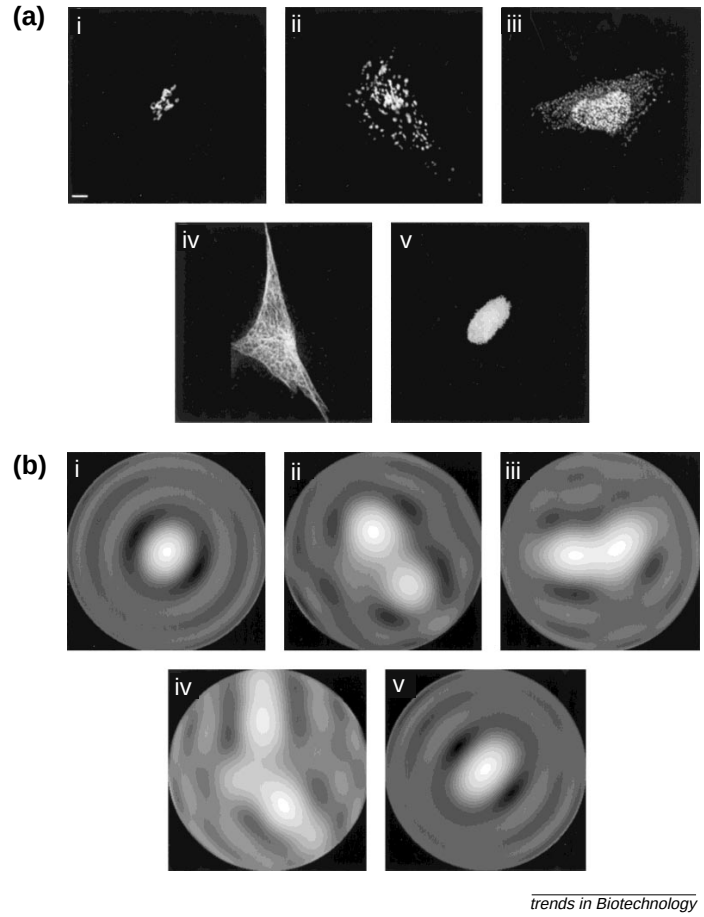

Figure 6

Computational methods can be used to extract information regarding the distribution of pixels in a micrograph and to reconstruct the original image. Boland *et al.*[41] computed the first 49 Zernike moments, which statistically describe the location of bright pixels in each image, of (**a**) fluorescence micrographs i–v and (**b**) reconstructed the images using these Zernike moments.

fractionation[44], as well as recent innovations, such as expression cloning[45] and display cloning[46].

Recently, large-scale transcription profiling has been used to identify specific metal ions as the targets of a set of active compounds identified in a reporter gene assay[23]. This indicates that the pattern of changes in gene expression that are observed in response to treatment with a small molecule might reveal the mechanistic basis for its activity. Furthermore, Marton *et al.*[47] demonstrated that the transcription profile of a small molecule is not observed in cells in which the target of the small molecule has been deleted. Treatment of yeast cells with 3-aminotriazole (3-AT), which inhibits the HIS3 gene product, caused more than 1000 changes in transcription. However, treatment of HIS3-deficient yeast cells with 3-AT resulted in no changes in transcription. Although the use of such a method requires high-throughput transcription profiling and is therefore not currently viable, it is probable that in the future this method will become a powerful strategy for unambiguously defining the molecular target of a small molecule.

Conclusions

Previous technical hurdles have prevented a systematic application of forward genetic-like studies in mammalian systems. However, using diverse chemical libraries, transcription profiling, reporter gene assays, cytoblots, and automated microscopy it will soon be possible to design a high-throughput chemical genetic screen for any biological process or disease state imaginable. As the available pool of organic compounds increases, more and more of these screens will yield fascinating and powerful reagents that act as conditional switches for affecting biological systems and disease states. Furthermore, as methods of target identification are improved, these compounds will allow rapid mapping of proteins to disease states and basic biological functions. Finally, the ability to screen directly for compounds with a desired property (e.g. those that suppress formation of a disease state) might become a powerful engine driving the development of new therapeutic agents. We can expect that both forward and reverse chemical genetic approaches will continue to provide a multitude of insights into the molecular basis of biological processes and human disease.

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New perspectives on the design of cytokines and growth factors

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A combination of molecular modelling, conventional epitope scanning and combinatorial techniques, such as phage display and DNA shuffling, has greatly improved our understanding of ligand–receptor interactions. It has therefore been possible to develop powerful cytokine–growth factor antagonists and new designer cytokines, with altered receptor specificities or with greatly enhanced biological activity. Recently, small circular peptides that mimic or block the effects of natural cytokines and growth factors have been developed; such small peptides are likely to open new avenues in therapeutics.

Epitopes are regions of proteins that are involved in noncovalent interactions with other proteins[1]. The identification of ligand epitopes responsible for binding to cognate receptors – and, conversely, identification of receptor epitopes that bind ligands – has been a major focus of recent cytokine research. This interest is largely motivated by the hope that a detailed understanding of such protein–protein interactions will lead to new therapeutic possibilities. Here, we focus on some outstanding contributions to this field and on potential future developments.

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Defining and exploiting receptor–ligand interactions

Modern recombinant-DNA (rDNA) techniques, and the cloning of cDNAs that code for cytokines and their receptors, allowed the modification of growth

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