High-throughput screening of small molecules in miniaturized mammalian cell-based assays involving post-translational modifications

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Background: Fully adapting a forward genetic approach to mammalian systems requires efficient methods to alter systematically gene products without prior knowledge of gene sequences, while allowing for the subsequent characterization of these alterations. Ideally, these methods would also allow function to be altered in a temporally controlled manner.

Results: We report the development of a miniaturized cell-based assay format that enables a genetic-like approach to understanding cellular pathways in mammalian systems using small molecules, rather than mutations, as the source of gene-product alterations. This whole-cell immunodetection assay can sensitively detect changes in specific cellular macromolecules in high-density arrays of mammalian cells. Furthermore, it is compatible with screening large numbers of small molecules in nanoliter to microliter culture volumes. We refer to this assay format as a 'cytoblot', and demonstrate the use of cytoblotting to monitor biosynthetic processes such as DNA synthesis, and post-translational processes such as acetylation and phosphorylation. Finally, we demonstrate the applicability of these assays to natural-product screening through the identification of marine sponge extracts exhibiting genotype-specific inhibition of 5-bromodeoxyuridine incorporation and suppression of the anti-proliferative effect of rapamycin.

Conclusions: We show that cytoblots can be used for high-throughput screening of small molecules in cell-based assays. Together with small-molecule libraries, the cytoblot assay can be used to perform chemical genetic screens analogous to those used in classical genetics and thus should be applicable to understanding a wide variety of cellular processes, especially those involving post-transitional modifications.

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Introduction

A genetic approach to analyzing biological systems is extremely powerful in that novel gene products involved in a biological process of interest can be identified. The genetic approach entails determining the phenotypic consequences of mutations in genes and ordering genes into functional pathways. Such an approach has been widely used in a large number of genetically tractable organisms including fruit flies, nematodes, yeast, plants, and even complex vertebrates such as zebrafish and mice [1]. Furthermore, the genetic approach can be subdivided into 'forward' genetics, which involves phenotype-based screening of random mutations, and 'reverse' genetics, which involves studying the phenotypic consequences of mutations in a known gene.

Methods for comprehensive genetic analysis of mammalian systems are currently limited [2]. With whole organisms such as mice, the space requirement and expense of large numbers of animals, their long generation time, small litter size and the difficulty inherent in identifying and mapping recessive mutations (mutations that only result in a phenotypic effect when all copies of a gene are altered) are problematic. In addition, many gene products are essential, redundant or expressed in a temporal- or tissue-specific manner. Tissue-culture systems can alleviate some of these problems and yet still provide a suitable model system for understanding mammalian physiological and developmental pathways [3,4]. Reverse genetic techniques that are compatible with these systems, such as the use of antisense constructs, ribozymes or gene targeting, require prior knowledge of gene sequences and are not widely applicable to novel gene discovery or large-scale 'forward genetic' phenotype-based screens [4–7].

Fully adapting a forward genetic approach to mammalian systems requires efficient methods to alter gene products systematically without prior knowledge of gene sequences, while still allowing the subsequent recovery and characterization of these alterations. Ideally, these methods should also allow the conditional alteration of a gene product, through both loss and gain of function, in a temporally

defined manner. In addition, it would be useful to be able to perform suppressor and enhancer screens, which seek to identify genes that, when mutated, suppress or enhance a previously identified phenotype of interest. The advantage of such screens, as compared with using wild-type conditions, is that the pathway is sensitized to further perturbation, rendering the mutations identified more relevant to the pathway of interest.

A forward chemical genetic approach (use of phenotype-based screens) using small molecules that alter protein function directly has the potential to overcome many of the current limitations in genetic analyses of mammalian systems. This process is akin to the generation of mutations in genes, but relies on small molecules, often in the form of a chemical library, as the source of perturbation. As a small molecule in a cell-based assay can alter specifically the function of a gene product from all copies of a gene, a small molecule can be used analogously to an inducible dominant or homozygous recessive mutation. These characteristics circumvent the difficulty of generating these types of mutations in mammalian systems. Also, just as mutation sites can identify functionally relevant coding sequences of genes, small molecules can identify functionally relevant residues of proteins, based on their mechanism of interaction [8]. We reason that, by using small-molecule libraries in an appropriate cell-based assay, it should be possible to identify novel gene products on pathways of interest, as well as novel biologically active small molecules from either natural sources or laboratory syntheses ([9] and the Schreiber group website, http://www-schreiber.chem.harvard.edu). This idea is supported by the existence of a wide variety of small molecules that cause a loss of function of their cognate targets, including kinases [10], phosphatases [11], membrane receptors [12], proteases [8], isoprenyl transferases [13] and polymerases [14]. To a lesser extent, small molecules that result in a gain of function in a protein of interest have also been discovered or invented [15–17].

Once a new small-molecule modulator of a gene product is discovered, a reverse chemical genetic approach (use of small molecules whose target is known) is possible. This entails using the small molecule as a tool to alter the function of the gene product and subsequently observing the phenotypic effects. For example, an inhibitor of an essential gene product can be used to eliminate conditionally that gene product's function. The demonstrated effectiveness of the reverse chemical genetic approach makes this an attractive option, but this approach is often unavailable because the vast majority of gene products currently lack a small-molecule partner. A forward chemical genetic approach is therefore required. With the aim of performing forward chemical genetic screens to discover new small-molecule partners, we sought to develop a miniaturized cell-based assay format that would be suitable for high-throughput screening. We report here the development of such a format and we highlight its applicability to a broad range of genetic-like screens in mammalian cells. This assay format is capable of detecting post-translational and biosynthetic events, in a high-throughput manner, without the use of engineered cell lines or radioactivity.

Results

A whole-cell immunodetection assay related to ELISAs and western blotting

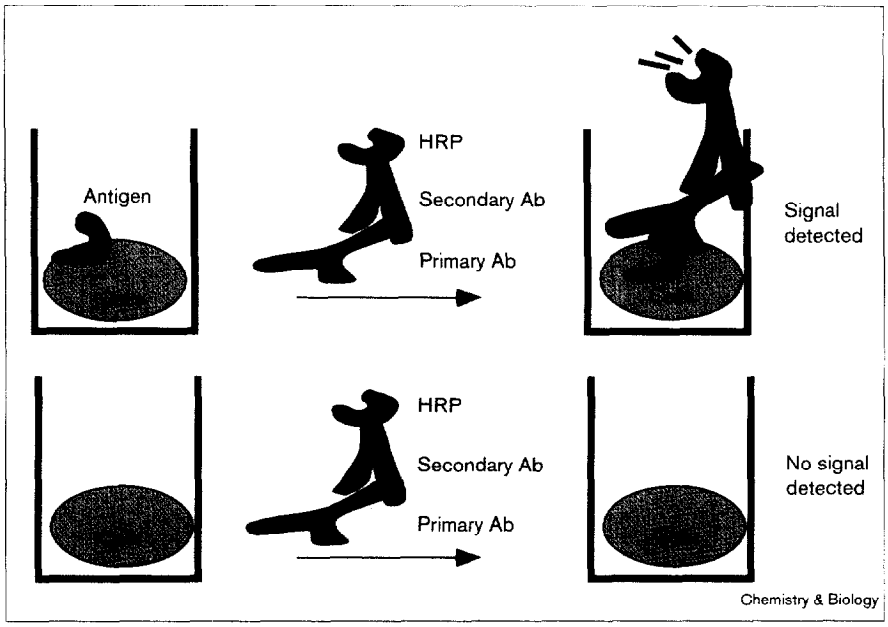
We have developed a high-throughput whole-cell immunodetection assay that is similar to both enzyme-linked immunosorbent assays (ELISAs) [18] and western blotting [19,20] (Figure 1). We refer to this technique as a cytoblot because, as in a western blot, an antibody is used to detect the presence of an antigen immobilized in the solid phase. In a cytoblot, a phosphorylated protein or other cellular molecule of interest is detected in whole, fixed cells on the bottom of a well using a specific primary antibody and a secondary antibody covalently linked to horseradish peroxidase (HRP) [21] (Figure 1). As in a western blot, the retention of this complex in the solid phase is detected by adding luminol, hydrogen peroxide and *p*-iodophenol (Figure 2a). The amount of antigen present is visualized directly on film using this chemiluminescent reaction (Figure 2a). Although antibodies are routinely used to analyze the cellular state of mammalian cells in conventional procedures such as western blotting, dot blotting, ELISAs, flow cytometry and immunocytochemistry, these procedures are not practical for high-throughput screening of small-molecule libraries in mammalian cells.

Detection of biosynthetic events in high-density arrays

We were interested in assaying for the extent of cellular biosynthetic events, such as DNA synthesis. We tested the ability of cytoblotting to detect changes in DNA synthesis by measuring the incorporation of 5-bromodeoxyuridine (BrdU, Figure 2a) in the presence or absence of transforming growth factor β (TGF-β), which arrests many cell types in the G_1 phase of the cell cycle [22,23]. BrdU is a thymidine analog in which the methyl group at the 5-position is replaced with bromine (Figure 2a). This analog is efficiently incorporated into DNA during DNA replication, and can be detected with an antibody raised specifically against this modified form [24–26]. We seeded 2000 mink lung cells, which are responsive to TGF-β [22], in each well of an opaque, white 384-well plate, treated the cells with varying concentrations of TGF-β for 16 h and then added 10 μM BrdU for 16 h. We found that TGF-β treatment effectively prevented BrdU incorporation and that background staining in the presence of TGF-β was negligible (Figure 2b). We also tested the ability of small-molecule cell-cycle-arresting agents to inhibit BrdU incorporation in this assay. We found that hydroxyurea, nocodazole, trapoxin and rapamycin efficiently prevented BrdU incorporation, but that FK506, as

Figure 1

Schematic of whole-cell immunodetection assay using chemiluminescent detection (a cytoblot). A cytoblot involves growing cells on the bottom of a well, fixing the cells and probing the cells for the presence of a particular antigen using a specific primary antibody in solution. A secondary antibody covalently linked to horseradish peroxidase (HRP) is added and the presence of the entire complex is detected through the chemiluminescent reaction caused by addition of luminol, hydrogen peroxide and an enhancer such as *p*-iodophenol. Ab, antibody.



expected, failed to prevent BrdU incorporation (Figure 2c) — cell-cycle progression and DNA synthesis are independent of FKBP12 and calcineurin, the protein targets of FK506, in mink lung epithelial cells (B.R.S. and S.L.S., unpublished observations). A cytoblot assay in this format can therefore be used to measure the extent of cell proliferation and to perform a chemical genetic screen for cell-cycle-arresting agents. Furthermore, the cell-cycle-arresting agents identified by this type of screen are likely to be the small-molecule partners of many of the gene products identified in classical yeast cell-division cycle (cdc) screens [27].

We tested whether these results could be extended to 1536- and 6144-well plates. We seeded mink lung cells with or without TGF-β in opaque, white 1536-well plates. TGF-β effectively prevented BrdU incorporation and background staining was negligible (Figure 3a). We then tested the anti-BrdU cytoblot assay in a plate containing 6144 arrayed 'nanowells' ([28]; R. King, personal communication). Again, TGF-β effectively prevented BrdU incorporation, background staining was negligible and individual wells were easily resolved, indicating that interwell crosstalk did not occur (Figure 3b).

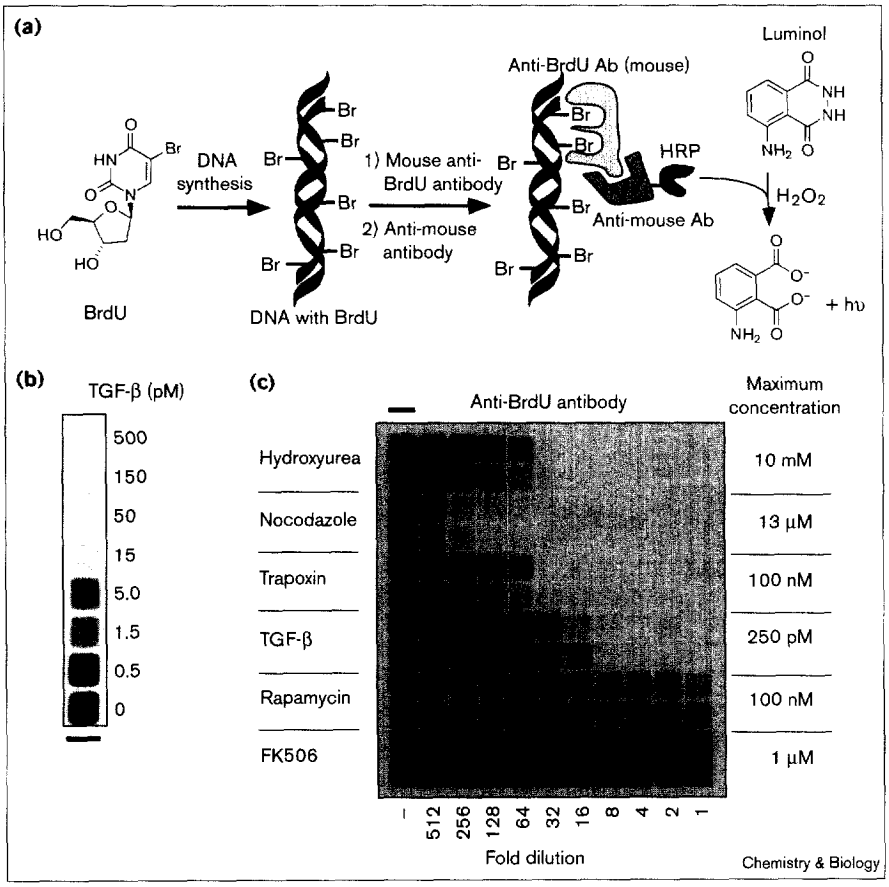
The cytoblot assay is compatible with a variety of cell types, both primary and transformed. We found that Mv1Lu mink lung epithelial cells (Figure 2c), A549 human lung carcinoma cells (data not shown), HeLaS3 human cervical carcinoma cells (data not shown) and mouse embryonic stem (ES) cells (Figure 3c) were compatible with the BrdU cytoblot. We found that pretreating the wells was important for allowing attachment of some cell lines. For example, pretreating a 384-well plate with 0.1% gelatin was the most efficient method of allowing ES cell attachment and growth (Figure 3c).

Although general inhibitors of DNA synthesis could be useful and interesting compounds [29], genotype-specific inhibitors of DNA synthesis would be even more useful. We obtained 192 marine sponge extracts from Professor P. Crews and M. Sanders, University of California, Santa Cruz (UCSC) and we tested these crude organic extracts (in duplicate rows) for their ability to inhibit BrdU incorporation in either $p53^{-/-}$ [30] or $p21^{Kip1-/-}$ [31] mouse embryonic fibroblasts (MEFs) using a BrdU cytoblot (Figure 3d). By overlaying the results of these experiments, we were able to identify extracts that were genotype-independent BrdU-incorporation inhibitors (black wells), $p21^{Kip1-/-}$ $p53^{+/+}$-specific BrdU incorporation inhibitors (red wells), and possibly some weak $p21^{+/+}$ $p53^{-/-}$-specific BrdU incorporation inhibitors (green wells).

Detection of post-translational modifications of endogenous proteins

As many biologically interesting and therapeutically important signaling pathways (including cell-cycle progression [32], gene expression [33], and determination of cell fate [34]) involve the reversible covalent modification of proteins as a means of post-translational regulation, it would be useful to screen small-molecule libraries for regulators of such modifications. Towards this end, we used an anti-acetylated histone H4 antibody in the cytoblot format to detect an increase in the acetylation of histone H4 in response to the histone-deacetylase inhibitors trapoxin

Figure 2



A cytoblot assay for DNA synthesis in high-density arrays of mammalian cells. **(a)** A schematic of an anti-BrdU cytoblot. The thymidine analog 5-bromodeoxyuridine (BrdU) is incorporated into the DNA of adherent cells that are actively replicating their DNA. BrdU is detected using a two-step antibody-binding procedure. The secondary antibody is conjugated to HRP. In the presence of luminol, hydrogen peroxide and *p*-iodophenol, light of wavelength 428 nm is generated. The light emission can be detected by exposing the plate to film. **(b)** A cytoblot can detect TGF-β's ability to prevent BrdU incorporation in mink lung epithelial cells. 2000 Mv1Lu mink lung epithelial cells were seeded in each well of a white, opaque 384-well plate. The cells were seeded in the indicated concentrations of TGF-β in 45 μl of 1% mink medium and incubated at 37°C with 5% CO_2. After 16 h, 5 μl of 100 μM BrdU in 1% mink medium was added to each well, for a final concentration of 10 μM BrdU. The cells were incubated at 37°C with 5% CO_2 for an additional 16 h and then an anti-BrdU cytoblot protocol was performed. **(c)** A cytoblot can detect the ability of numerous anti-proliferative agents to inhibit BrdU incorporation. Anti-BrdU cytoblots were performed as in (b) on cells treated for 43 h with the indicated concentrations of the anti-proliferative agents shown. BrdU treatment was for 7 h. Scale bars, 4 mm.

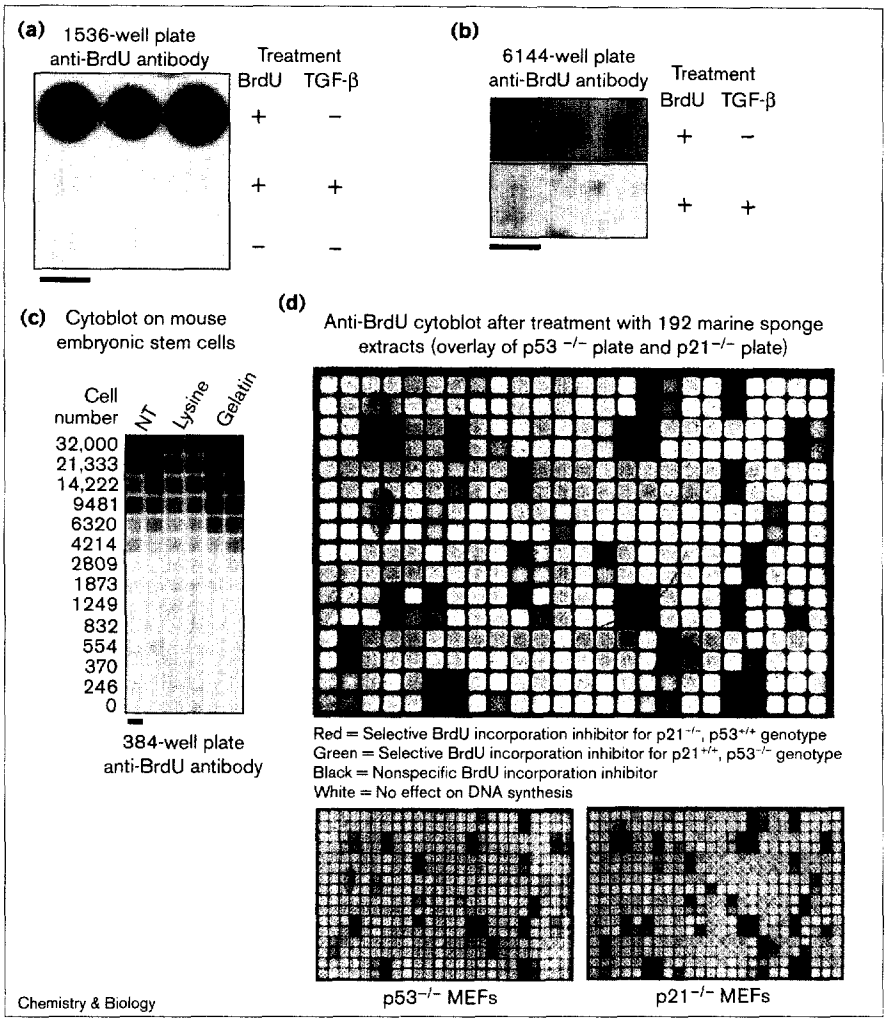
and trichostatin [35]. Treatment of A549 cells with trapoxin or trichostatin, but not other cell-cycle-arresting agents, caused an increase in acetylation of histone H4 (Figure 4a). An identical cytoblot in HaCaT cells [36], which do not upregulate p21 in response to trichostatin A (C. Grozinger, C. Hassig and S.L.S., unpublished observations), did not result in an increase in histone H4 acetylation (Figure 4a). As few as 500 cells could be detected with the anti-acetylated histone H4 antibody after just a 4 h treatment (Figure 4b).

Histone H3 and nucleolin are both phosphorylated in mitosis and antibodies against these phospho-epitopes have been used to detect mitotic cells [37–39]. TG-3, a monoclonal antibody that recognizes the phosphorylated form of nucleolin, has previously been used to identify novel small molecules from natural-product extracts that are capable of arresting cells in mitosis (M. Roberge and R. Anderson, personal communication) or inhibiting the DNA damage-induced G_2 checkpoint [40]. Here we extend the applicability of these antibodies as markers of mitosis and as tools for screening for anti-mitotic compounds in the cytoblot format. We treated cells with either the microtubule inhibitor nocodazole (Figure 4c,d) or other anti-mitotic agents (data not shown), and then used these antibodies to assay for mitotic cells. Both antibodies were capable of detecting mitotic cells (Figure 4c,d). With an anti-phosphonucleolin cytoblot we were able to detect as few as 500 cells in 250 nl cell culture in a 6144-well plate, after an 8 h nocodazole treatment (Figure 4e).

The use of these antibodies in the cytoblot format enables screening of small-molecule libraries for direct inhibitors and activators of histone deacetylases and acetyl transferases, as well as nucleolin and histone H3 kinases and phosphatases. Alternatively, one can use the alterations in acetylated histone H4, phosphohistone H3 and phosphonucleolin levels as more general markers of the cellular state, allowing one to screen for small molecules that indirectly induce these molecular changes (S.J.H., A. You and S.L.S., unpublished observations).

In order to screen rapidly the many hundreds of thousands or millions of compounds that can be synthesized using modern split-pool synthesis [41], we need a throughput even greater than that obtained using 384-well plates. We are currently optimizing methods of *en masse* compound delivery to 1536- and 6144-well plates so

Figure 3



BrdU incorporation can be efficiently detected with cytoblots in high-density plates using multiple cell lines. **(a)** 500 Mv1Lu mink lung epithelial cells were seeded in 2 µl in each well of a white, opaque 1536-well plate (Corning/Costar). The cells were seeded with or without 400 pM TGF-β in 2 µl of 1% mink medium and allowed to incubate at 37°C with 5% CO_2. After 24 h, 0.5 µl 50 µM BrdU in 1% mink medium was added to the indicated wells, yielding a final concentration of 10 µM BrdU. The plate was incubated at 37°C with 5% CO_2 for an additional 12 h and then an anti-BrdU cytoblot protocol was performed. Scale bar, 1.5 mm. **(b)** Mv1Lu cells were seeded on plasma-cleaned 6144-well polydimethylsiloxane (PDMS) plates (R. King, personal communication) at a density of 100,000 cells/ml in 1% mink medium. After 6.5 h the cells had attached and new medium with or without 500 pM TGF-β1 was added to the plates and the excess removed, leaving approximately 250 nl per well. The cells were incubated for 18.5 h at 37°C with 5% CO_2, then the medium was washed out and new 1% mink medium with 10 µM BrdU was added. After 90 min an anti-BrdU cytoblot was performed. Scale bar, 1 mm. **(c)** The indicated number of mouse embryonic stem cells were seeded in 90 µl of ES medium (DMEM, 15% characterized FBS, Hyclone, 0.1 mM β-mercaptoethanol, Sigma, 0.1 mM nonessential amino acids, GibcoBRL, 100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, 2 mM glutamine, GibcoBRL, 250 U/ml leukemia inhibitory factor, ESGRO, GibcoBRL) on a 384-well plate that had been precoated with nothing (NT), poly-L-lysine (lysine) or 0.1% gelatin (gelatin). The cells were incubated for 24 h at 37°C with 5% CO_2 and then BrdU was added to a final concentration of 10 µM. After 12 h an anti-BrdU cytoblot was performed. **(d)** 2500 mouse embryonic fibroblasts (MEFs; $p53^{-/-}$ or $p21^{Kip1-/-}$) were seeded in MEF medium in 384-well white plates and cultured overnight at 37°C with 5% CO_2. Marine sponge extracts were pin-transferred (1.19 mm 96 pin array, V&P Scientific) in duplicate rows from 10 mg/ml dimethylsulfoxide (DMSO) stock solutions into 40 µl MEF medium for both cell lines. After 24 h BrdU was added to a final concentration of 10 µM and the cells were cultured for an additional 8.5 h. A BrdU cytoblot was performed on each plate. The film images of the results were scanned into Photoshop 5.0 (Adobe) and converted to inverse white/red and white/green color scales and merged, with one layer 50% transparent.

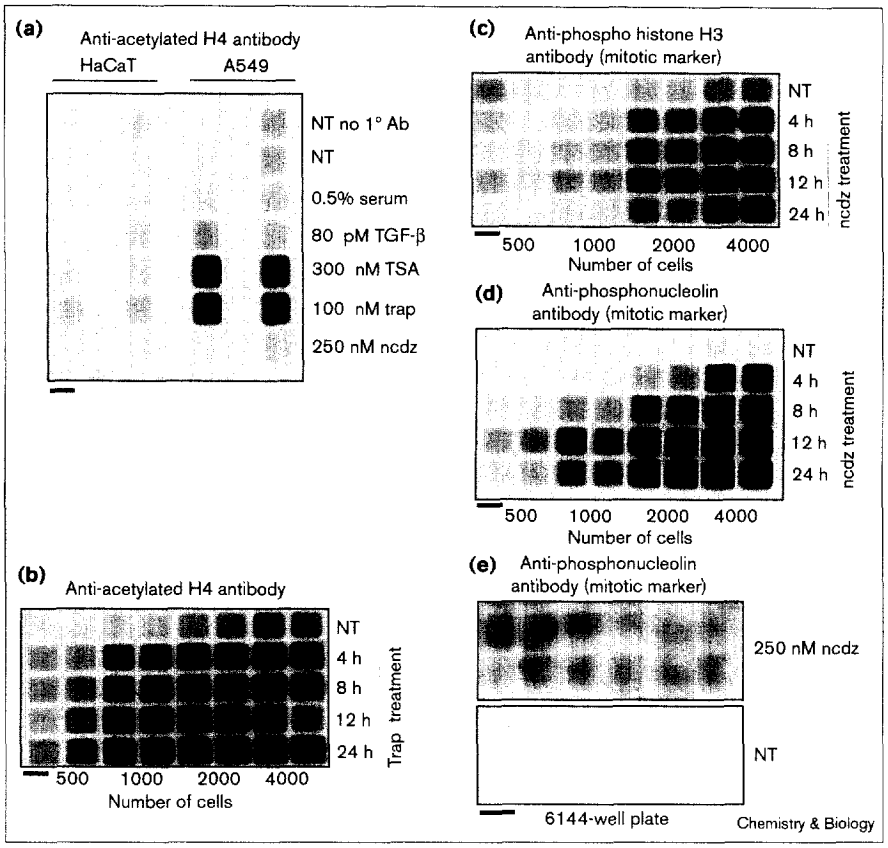
that we can perform BrdU, phosphonucleolin and other cytoblot screens in these formats (B.R.S., S.J.H. and S.L.S., unpublished observations).

A small-molecule suppressor of rapamycin

With the aim of developing a general approach analogous to that of suppressor and enhancer screening in classical genetics, we would like to screen for small molecules that can modify the effects of other chemical or genetic alterations. To demonstrate that this is possible and to show that cytoblots can detect small-molecule suppressors of anti-proliferative agents, we tested the ability of FK506 to act as a suppressor of rapamycin.

FK506 and rapamycin share a common binding protein, the 12 kDa FK506- and rapamycin-binding protein (FKBP12) [42]. The FKBP12–rapamycin and FKBP12–FK506 complexes target different proteins, FKBP12-rapamycin associated protein (FRAP) and calcineurin, respectively (Figure 5a) [11,43]. Simultaneous treatment of mink lung cells with rapamycin and excess FK506 prevents rapamycin from binding FKBP12 and thereby prevents rapamycin-induced growth arrest (Figure 5a; B.R.S. and S.L.S., unpublished observations). In a cytoblot format, this suppressor effect of FK506 should be manifested as the ability of cells to incorporate BrdU in the presence of both rapamycin and FK506 (Figure 5a). Indeed, an excess of FK506 suppressed the

Figure 4



Cytoblot assays for the accumulation of hyperacetylated histone H4, phosphonucleolin and phosphorylated histone H3. **(a)** 4000 A549 human lung carcinoma cells were seeded in 40 µl in a white 384-well plate, allowed to attach overnight and then either not treated (NT), or washed once and treated with 0.5% serum, 80 pM TGF-β, 300 nM trichostatin A (TSA), 100 nM trapoxin (trap) or 250 nM nocodazole (ncdz) and incubated for 24 h at 37°C with 5% CO_2 in a final volume of 50 µl. A cytoblot was performed and the presence of the hyperacetylated form of histone H4 was detected using anti-acetylated H4 antibody and a secondary antibody conjugated to HRP. **(b)** Human HeLaS3 cells were seeded in 40 µl in a white 384-well plate, allowed to attach overnight and either not treated (NT) or treated with trapoxin at a final concentration of 100 nM for the times indicated and incubated at 37°C with 5% CO_2 in a final volume of 50 µl. A cytoblot was performed as in (a). **(c)** A549 cells were seeded in 40 µl in a white 384-well plate, allowed to attach overnight and either not treated (NT) or treated with nocodazole (ncdz) at a final concentration of 250 nM for the times indicated and incubated at 37°C with 5% CO_2 in a final volume of 50 µl. A cytoblot was performed and the presence of the phosphorylated form of histone H3 detected using anti-phospho histone H3 mitosis marker and a secondary antibody conjugated to HRP. **(d)** HeLaS3 cells were seeded in 40 µl in a white 384-well plate, allowed to attach overnight and either untreated (NT) or treated with nocodazole (ncdz) at a final concentration of 500 nM for the times indicated and incubated at 37°C with 5% CO_2 in a final volume of 50 µl. A cytoblot was performed and the presence of the phosphorylated form of nucleolin detected using the TG-3 antibody and a secondary antibody conjugated to HRP. **(e)** A sample of wells from a 6144-well plate were collectively incubated in 1 ml of A549 cells (500 cells per well) and were either not treated (NT) or treated with nocodazole (ncdz) at a final concentration of 500 nM and incubated for 24 h at 37°C with 5% CO_2 in a final volume of 50 µl. A cytoblot was performed. Scale bars (a–d), 4 mm; (e), 1 mm.

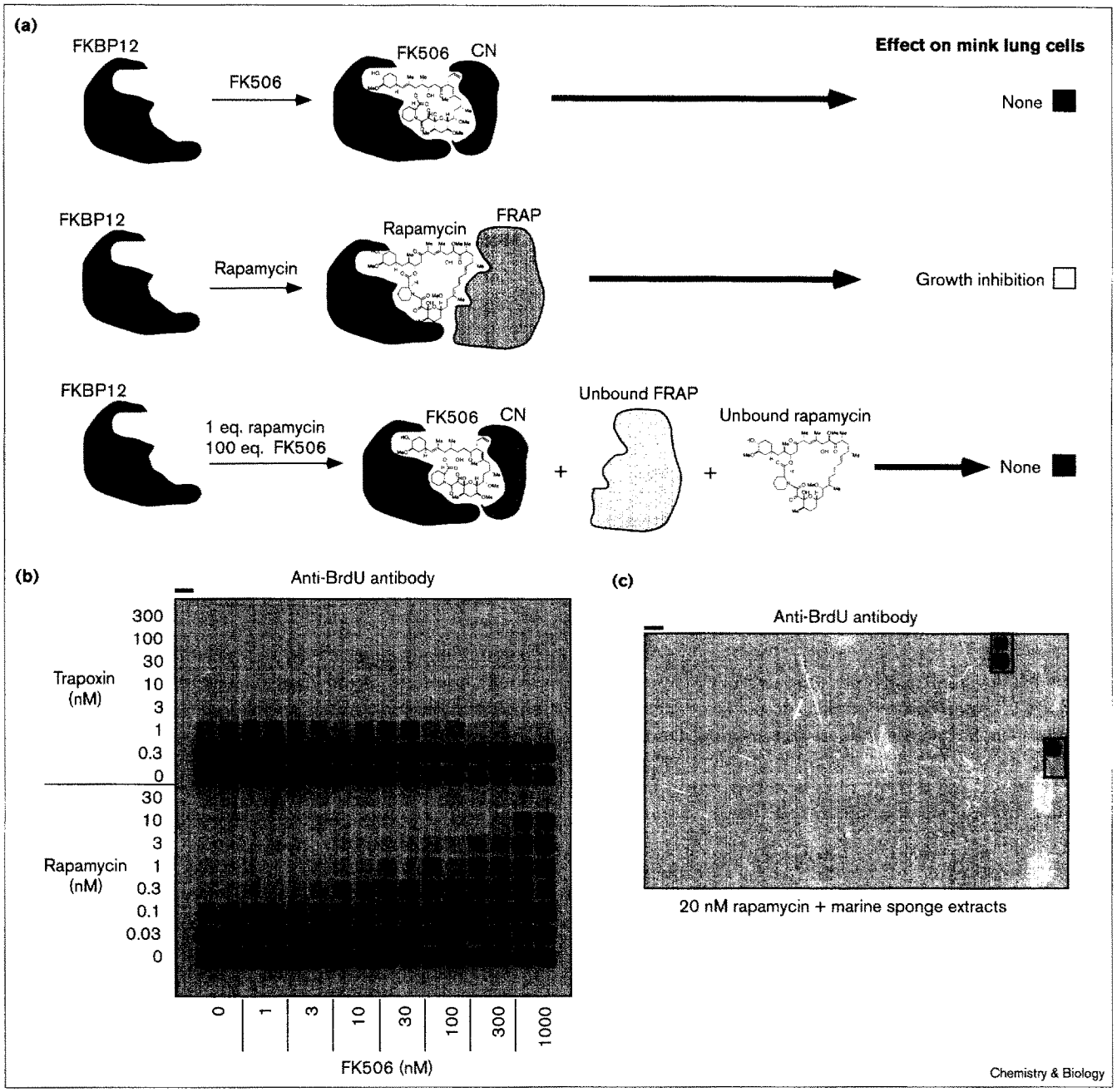
anti-proliferative effect of rapamycin in an anti-BrdU cytoblot (Figure 5b). FK506 should not, however, and did not, suppress the anti-proliferative activity of the unrelated molecule trapoxin, which does not require FKBP12 for its anti-proliferative effects (Figure 5b) [35].

To demonstrate that it is possible to identify natural products that act as suppressors of anti-proliferative agents, we screened 192 marine sponge extracts for suppressors of the anti-proliferative effect of rapamycin. We identified two crude organic extracts that allowed mink lung cells to incorporate BrdU in the presence of 20 nM rapamycin (Figure 5c), a concentration that otherwise prevents BrdU incorporation in these cells (Figure 5b). The extracts were tested in duplicate rows and the two hits are shown in red boxes (Figure 5c). A third extract with weak suppressor activity was visible upon longer exposure to film (data not shown). All three hits were confirmed by retesting the extracts in duplicate. These active extracts were generated from Indo-Pacific marine sponges, collected by the Crews group. Two of these samples came from sponges in the family Petrosiidae, and the third originates from a specimen most closely resembling *Callyspongia ramosa*. All three sponges belong to the order Haplosclerida. Taxonomic identification of the source organisms and further chemical analysis of the active extracts are now underway. In collaboration with Crews and Sanders, we hope to test further these extracts in this suppressor assay. This suppressor-screening strategy can also be applied to other anti-proliferative or cytostatic proteins and small molecules such as TGF-β, hydroxyurea, mimosine, lovastatin, nocodazole, benomyl and depudicin, as well as DNA-damaging agents such as mitomycin, bleomycin, cisplatin, ultraviolet light and gamma irradiation [29].

Screens for small-molecule suppressors of other cell-cycle arresting agents

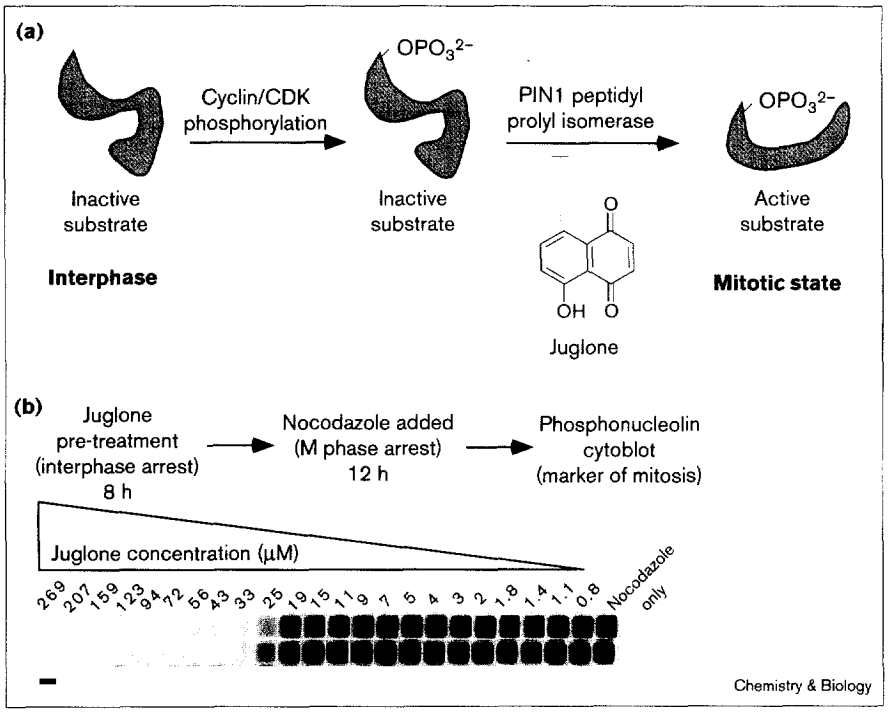
We attempted to extend the above demonstration of small-molecule suppressors, as well as the previous use of TG-3 in monitoring the G_2 checkpoint [40], to other cytoblot

Figure 5



Genetic-like screens using small molecules. **(a)** A schematic of the ability of excess FK506 to suppress the anti-proliferative effect of rapamycin. Excess FK506 binds all available FKBP and thereby prevents rapamycin from binding FKBP. Rapamycin cannot bind FRAP, and therefore does not inhibit proliferation, in the absence of FKBP. **(b)** FK506 suppresses the anti-proliferative activity of rapamycin, but not trapoxin. 2000 6F mink lung epithelial cells (6F cells, a stable cell line in which the small molecule FK1012 activates TGF-β signaling [61], are more responsive to the growth inhibitory effects of rapamycin than the parental Mv1Lu cell line [B.R.S. and S.L.S., unpublished observations]) were seeded in each well of a white, opaque 384-well plate. The cells were seeded in the indicated concentrations of rapamycin or trapoxin in 40 µl of 1% mink medium and immediately 40 µl of 2× stocks of the indicated concentrations of FK506 was added to each well and then the cells were allowed to incubate at 37°C with 5% CO_2. After 24 h, 9 µl of 100 µM BrdU in 1% mink medium was added to each well, for a final concentration of 10 µM BrdU. The cells were incubated at 37°C with 5% CO_2 for an additional 16 h and then an anti-BrdU cytoblot protocol was performed. **(c)** Identification of crude organic marine sponge extracts that are capable of suppressing rapamycin's anti-proliferative effect. 2000 6F mink lung epithelial cells were seeded in 50 µl of 1% mink media containing 20 nM rapamycin in each well of a white 384-well plate. 192 marine natural product extracts (10 mg/ml stock solution in DMSO) were assayed in duplicate rows by transferring approximately 50 nl to each assay well using a 96 pin array (V&P Scientific, catalog number VP409). After 49 h, 10 µl of 6X BrdU was added to each well, yielding a final concentration of 10 µM BrdU. After 13 h an anti-BrdU cytoblot was performed. Scale bars, 4 mm.

Figure 6



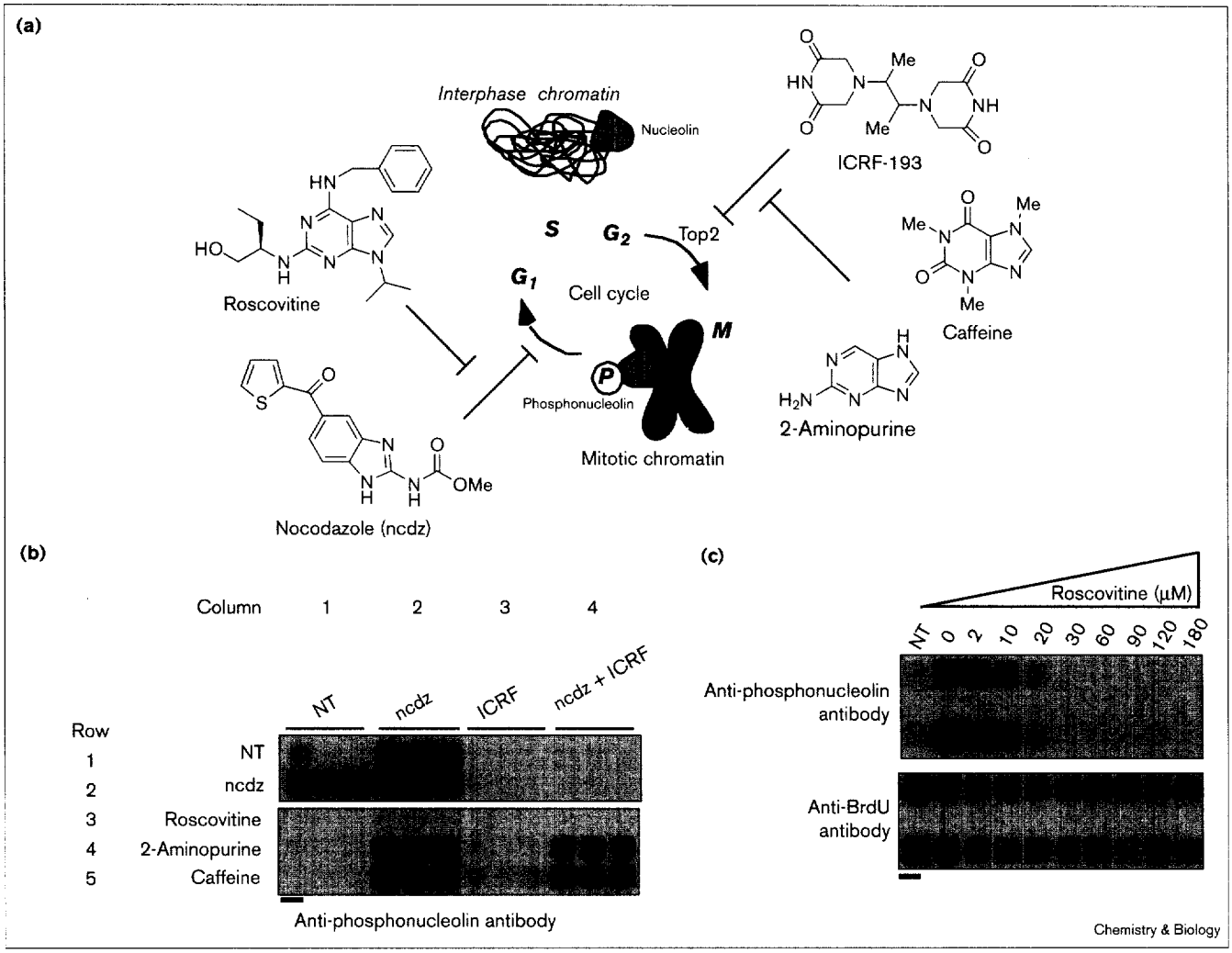
The ability of anti-proliferative agents such as juglone to suppress the effects of nocodazole-induced mitotic arrest can be detected in a cytoblot. **(a)** Schematic of the ability of juglone to inhibit the effects of the *cis/trans* peptidyl-prolyl isomerase Pin1. The activity of Pin1 is required for proper mitotic progression. If inhibition of Pin1 activity results in an interphase arrest, juglone treatment should prevent the entry of cells into mitosis [46,47]. **(b)** A549 human lung carcinoma cells were seeded at a density of 4000 cells in 40 μl of DMEM+ in each well of a white, opaque 384-well plate and incubated overnight at 37°C with 5% CO_2. Cells were either untreated or pretreated with juglone at the indicated concentrations for 8 h. Subsequently, nocodazole at a final concentration of 250 nM was added to all wells in a final volume of 50 μl and the cells incubated for a further 12 h. A phosphonucleolin cytoblot was performed. Equivalent concentrations of methanol had no effect on phosphonucleolin levels (data not shown). Scale bar, 4 mm.

assays. We took advantage of the fact that treatment of cells with any small molecule that arrests cells outside of mitosis will suppress the ability of nocodazole to arrest cells in mitosis. Recently, the natural-product juglone [44] was demonstrated to be a selective, covalent inhibitor of Pin1 *in vitro*. Pin1 is a member of the parvulin family of peptidyl-prolyl *cis/trans* isomerases (PPIases), whose activity has been implicated in progression through mitosis in *Xenopus* and yeast (Figure 6a) [45–47]. We demonstrated that pretreatment of cells with > 25 μM juglone prevents accumulation of phosphonucleolin upon nocodazole treatment (Figure 6b). Similar results were obtained using trapoxin and camptothecin (data not shown), both of which arrest cells outside of M phase.

To demonstrate that it is possible to find small-molecule suppressors of G_2-arresting agents (i.e. small molecules that allow entry into mitosis in the presence of a G_2-arresting agent), we tested the ability of purine analogs to suppress the G_2 arrest caused by the topoisomerase II (Top2) inhibitor ICRF-193 [48] (Figure 7a). These purine analogs are known to be nonspecific competitive inhibitors of 5′-adenosine-triphosphate binding and therefore are likely to inhibit a wide range of kinases in the cell [29,48]. As expected, cells treated with nocodazole arrested in mitosis and therefore contained substantial phosphonucleolin (Figure 7b, row 1, column 2). The simultaneous addition of either ICRF-193 (row 2, column 3), or roscovitine (row 3, column 2), prevented this mitotic arrest, presumably by arresting the cells in interphase. The simultaneous addition of 2-aminopurine or caffeine, however, prevented the arrest in response to ICRF-193 and allowed cells to accumulate in mitosis in the presence of nocodazole (rows 4–5, column 4). As expected, nocodazole was required for the accumulation of cells in mitosis (rows 4–5, column 3). It is, therefore, possible to screen for inhibitors of cell-cycle-arresting agents using both anti-BrdU and anti-phosphonucleolin antibodies in the cytoblot format.

The juglone experiment (Figure 6b) demonstrates that it is possible to find molecules that suppress the effects of nocodazole treatment by preventing entry into mitosis. It should also be possible to find small molecules that induce exit from mitosis, resulting in a reduction in phosphonucleolin levels. As the nocodazole-induced arrest of cells in mitosis requires cyclin-dependent kinase (CDK) activity for the maintenance of the spindle-assembly checkpoint, inhibition of CDK activity should suppress nocodazole-induced mitotic arrest (Figure 7a). We pretreated cells with nocodazole for 14 h to arrest cells in mitosis (resulting in the accumulation of phosphonucleolin) and then added increasing amounts of roscovitine, a small-molecule inhibitor of CDKs [49], for 8 h. As a result of roscovitine treatment, cells exited from the nocodazole-induced arrest, as measured by the disappearance of phosphonucleolin (Figure 7c) and flow cytometry (data not shown). On the basis of a similar anti-BrdU cytoblot (Figure 7c), however, these cells did not begin to incorporate BrdU, indicating that the ability to proliferate was not restored by roscovitine treatment. This is not surprising, as CDK activity is required for S-phase progression.

Figure 7



Cytoblots can be used to screen for small-molecule suppressors of anti-proliferative agents using the presence of phosphonucleolin, and for small molecules that induce exit from mitosis using the absence of phosphonucleolin. **(a)** Schematic of the topoisomerase II (Top2)-dependent change in chromatin conformation required for entry into mitosis and the ability of caffeine and 2-aminopurine to suppress the effects of the Top2 inhibitor ICRF-193. **(b)** The ability of caffeine and 2-aminopurine to suppress the DNA-damage-independent, topoisomerase inhibitor-induced G2-checkpoint arrest [48] can be detected in a cytoblot. A549 human lung carcinoma cells were seeded at a density of 4000 cells in 40 μl of DMEM+ in each well of an opaque 384-well plate and incubated for 24 h at 37°C with 5% CO_2. Cells were then either left untreated (NT) or treated with 250 nM nocodazole (ncdz), 20 μM roscovitine, 1 mM 2-aminopurine or 2 mM caffeine, and simultaneously treated with either DMEM+ (NT), 250 nM nocodazole (ncdz), 14 μM ICRF-193 or both 250 nM nocodazole and 14 μM ICRF-193 (ICRF-193 + ncdz) in a final volume of 50 μl. Cells were then incubated for 18 h at 37°C with 5% CO_2 and a phosphonucleolin cytoblot was performed. **(c)** HeLaS3 cells were seeded at a density of 4000 cells in 40 μl of DMEM+ in each well of an opaque 384-well plate and incubated for 24 h at 37°C with 5% CO_2. Cells were either left untreated or treated with 554 nM nocodazole for 14 h to arrest cells in mitosis. Roscovitine was then added to the final concentrations indicated in a final volume of 50 μl and cells were incubated for 4 h at 37°C with 5% CO_2. Finally, BrdU was added to a final concentration of 10 μM to those wells that were assayed for BrdU incorporation, and the cells incubated for an additional 6 h at 37°C with 5% CO_2. BrdU and phosphonucleolin cytoblots were performed.

Discussion

There are several existing approaches for the discovery of biologically active small molecules. In one approach, small molecules are designed to interact with the active sites of proteins whose structures have been elucidated [50]. In a second approach, screening methods are developed to identify novel protein–small-molecule interactions [51,52]. These and related methods can be used to detect specific protein–ligand interactions using the effects of recombinant proteins or small molecules on the proliferation of cells, without concern for the specific cellular pathways involved [53,54]. Neither of these approaches can identify novel pathway components, however. In contrast, appropriately designed cell-based assays targeting specific

pathways can probe a cell's complete complement of gene products simultaneously.

To date, most cell-based assays have used reporter genes as an indicator of cellular activity [55]. Reporter genes, however, restrict the detection of cellular processes to those that cause changes in transcriptional events. It would be useful to screen directly for small molecules that can affect post-translational events such as protein glycosylation, methylation, lipidation, isoprenylation, ubiquitination, phosphorylation and acetylation. The cytoblot format is capable of detecting such post-translational events, given the existence of an appropriate antibody, and therefore should be a useful tool for cell-based screens involving such modifications. In addition, in contrast to reporter-gene-based assays, cytoblots can detect changes in protein concentration directly without the need to engineer a stable reporter-gene-expressing cell line. This facilitates simultaneous assaying of transformed or primary cell lines that are from different tissue types or from different genetic backgrounds (e.g., comparing $p21^{Kip1-/-}$ with $p53^{-/-}$ mouse embryonic fibroblasts [Figure 3d]). It is also possible to grow, and perform cytoblot assays on, mouse embryonic stem cells (Figure 3c) using BrdU. It should therefore be possible to use other antibodies against markers of differentiation in ES cells, thereby allowing screens for differentiation-inducing agents.

The changes in mRNA levels in a cell that result from treatment with a small molecule can be used as a fingerprint [56]. Although mRNA detection in this manner is a powerful tool, many cellular events, including all post-translational events, cannot be detected with this method. Cytoblots can provide a post-translational profile of the effect of a small molecule on cells, however, and therefore can be used to classify compounds functionally.

The demonstrated ability of cytoblots to report on induced post-translational events makes them a valuable tool for chemical genetic screening. There are, however, several important differences between small molecules and mutations as the source of phenotypic variation in a chemical-genetic or genetic screen. First, unlike most mutagenic methods, the use of small-molecule libraries will not generally produce heritable alterations in genes. Because a small molecule can generally be added and removed from an experiment at will, this means that the perturbations induced by small molecules are generally conditional and reversible. Second, it is large numbers of small molecules and not mutations that must be generated in order to perturb potentially the complete complement of cellular gene products. Third, determining which gene product is phenotypically altered in a cytoblot assay will require identifying the target of the small molecule, as opposed to the mapping of a mutation or sequencing of a gene.

The use of small-molecules to alter gene-product function already permeates many routine aspects of biology including the synchronization of cells, growth selection of recombinant bacteria or engineered cells, and inhibition of proteases and phosphatases during biochemical purification of proteins to maintain protein integrity. Traditionally, the source of such useful small molecules has been extracts containing natural products [57]. The advent of modern split-pool synthesis now adds to this repertoire complex 'natural-product-like' libraries, the products of laboratory syntheses, as a source of small molecules to be screened for novel compounds with biological activity [41]. These libraries will allow one to access a vast array of chemical structures that have not been selected by nature over time and therefore could allow the identification of structurally distinct and novel regulators of gene products for which there exist no natural-product partners. In order to screen the hundreds of thousands or millions of molecules in such libraries, in analogy to a geneticist's need to screen such numbers of mutations to achieve saturation of the genome, an appropriate assay format is required. Although the conventional format for high-throughput screening is a 96-well plate, this throughput will probably be insufficient to screen very large libraries. Moreover, in order to achieve a reasonable concentration of limited reagents and to minimize expense, space requirements, reagent consumption and time, small assay volumes must be used. To satisfy this need, commercial vendors have developed 384-well and 1536-well tissue culture plates. In addition, a miniaturized tissue-culture plate containing 6500 'nanowells' with 1 mm diameter wells was recently reported [28].

These small-assay formats themselves impose a number of constraints on assay design. First, culturing mammalian cells in small volumes can be problematic because of nutrient depletion, toxin accumulation and evaporation (B.R.S. and S.L.S., unpublished observations). Second, detection of the limited number of cells that can be grown in nanoliter volumes requires advances in optical imaging technology, including the ability to resolve individual wells and to detect weak signals. Finally, in dense arrays, with the current technology, sequential detection of a large number of wells is unacceptably slow, meaning that parallel detection is necessary. We have demonstrated here that cytoblots, which can be detected rapidly in parallel, are compatible with culturing cells in nanoliter volumes in dense arrays. Cells cultured in 384-, 1536- and 6144-well plates still retained the ability to replicate their DNA, as evidenced by BrdU incorporation (Figures 2b,c, 3a–c). This indicates that normal cellular proliferation is occurring on the time scale of the cytoblot assay and that cells cultured in nanowells remain sufficiently viable. As well, the use of chemiluminescent detection allows parallel imaging of wells regardless of the density, and provides an inexpensive, visual and quantifiable record of the assay results.

Once a small-molecule suppressor or enhancer of a phenotype is discovered, the identification of its protein target will be a high priority, and will rely upon target identification methods that employ the small molecule as bait. One method involves preparing radiolabeled derivatives of the small molecule and determining the molecular targets that are labeled, perhaps covalently, by these radioactive probes [58]. A second method identifies the target protein biochemically, by fractionating cellular extracts with an affinity matrix covalently modified with the biologically active small molecule [35]. A third method uses a 'three-hybrid' transcriptional activation system, which anchors a derivative of the active ligand for display against a library of cDNAs fused to a transcriptional activation domain [51,59]. Finally, expression cloning can be used to test for the presence of the target within a small pool of proteins [60].

It is worth comparing cytoblots, western blots and dot blots to see their relative advantages. Unlike a western blot, a cytoblot does not reveal the molecular weight of the target antigen and therefore cannot easily distinguish between specific and nonspecific reactivity. Therefore, when performing a cytoblot with an antibody that has not previously been characterized, it is important to confirm that the reactivity is due to binding to the desired target antigen. It might be possible to perform cytoblots against small-molecule second messengers in the cell that would not be visible in a western blot. It might also be possible to distinguish between subcellular localizations with a cytoblot but not a western blot or dot blot, on the basis of accessibility of antigens to the primary antibody or selective dissolution of the contents of the cell. Cytoblots are also preferable to western and dot blots in the analysis of large numbers of compounds.

Cytoblots could also be useful alternatives to western and dot blots in the routine characterization of recombinant protein expression levels, and the extent of activation of signaling pathways. These applications, as well as antibody screening and optimization are easily realized in the cytoblot format because of its high-throughput nature, minimal use of reagents, and short time requirement. Finally, in comparing cytoblots and dot blots, cytoblots have the advantage of preserving cellular architecture, as well as not requiring cell lysis and subsequent lysate transfer, which are potentially rate-limiting and variable steps when dealing with large numbers of samples. Cytoblots could therefore become a useful new tool in the arsenal of chemical geneticists and molecular biologists.

Significance

To date, most cell-based assays have used reporter genes, which detect transcriptional events, as an indicator of cellular activity. New cell-based assays that are both compatible with high-throughput screening and capable of detecting post-translational events are desirable. We have developed a miniaturized cell-based assay format, referred to as a cytoblot, that visualizes changes in biosynthetic processes, such as DNA synthesis, and post-translational protein modifications, such as acetylation and phosphorylation. Such post-translational alterations cannot be detected with reporter gene assays. The cytoblot assay does not require the generation of stably transfected cell lines, the use of radioactivity or the use of sensitive optical imaging systems, but simply uses a specific primary antibody directed against the endogenous cellular molecule of interest. High-throughput screens can therefore be performed simply using plates, cells, antibodies and film. The cytoblot assay, which can be performed in 384-, 1536- or 6144-well plates, can be used in a genetic-like approach to understanding cellular pathways in mammalian cells. Novel biologically active chemicals can be classified functionally by using cytoblots to simultaneously detect a variety of biosynthetic and post-translational changes in a cell. Finally, cytoblots could be convenient and efficient alternatives to western blotting, dot blotting and reporter-gene assays in the routine analysis of the function and presence of recombinant proteins in mammalian cells.

Materials and methods

Cell culture

Mv1Lu mink lung epithelial cells were obtained from the American Type Culture Collection (ATCC, catalog number CCL64) and cultured at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 100 μM each of the amino acids alanine, aspartate, glutamine, glycine, asparagine and proline (GibcoBRL) (referred to as 10% mink medium). Clone 6f mink lung epithelial cells, reported previously [61], were cultured in 10% mink medium supplemented with 700 μg/ml G418 sulfate (GibcoBRL). HeLaS3 cervical carcinoma and A549 lung carcinoma cells were cultured at 37°C with 5% CO_2 in DMEM with 10% FBS, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 2 mM glutamine (GibcoBRL). $p53^{-/-}$ and $p21^{Kip1-/-}$ MEFs were grown in DMEM with 10% fetal calf serum (GibcoBRL), 2 mM glutamine (GibcoBRL), 100 μM of the nonessential amino acids (GibcoBRL), 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (MEF medium).

384-well anti-BrdU cytoblot

2000 cells were seeded in each well of a white, opaque 384-well plate (Nalge Nunc) using a Multidrop 384 liquid dispenser (Labsystems). The cells were seeded in 40–50 μl of 1% medium containing the reagent of interest. After 16–36 h at 37°C with 5% CO_2, BrdU was added, using the Multidrop 384, from a 10× stock in medium (prepared from a 1000× stock in phosphate-buffered saline (PBS), pH 7.4) to a final concentration of 10 μM BrdU. The cells were incubated for an additional 4–16 h at 37°C with 5% CO_2. The plate was cooled on ice for 15 min and kept under aluminum foil to minimize light exposure for all remaining operations. The supernatant was removed from each well with a 24-channel wand (V&P Scientific), used throughout the protocol for aspiration, attached to a house vacuum source. The cells were fixed with 50 μl of cold (4°C) 70% ethanol/30% PBS, incubated for 1 h on ice, washed with 90 μl of cold (4°C) PBS and then 25 μl of 2 M HCl / 0.5% Tween 20 / ddH2O was added. The cells were incubated at room temperature for 20 min, then washed with 90 μl of 10% 2 M NaOH / 90% Hank's Balanced Salt Solution (HBSS, GibcoBRL), twice with 90 μl of HBSS, and once with 75 μl of PBSTB (PBS, 0.1% Tween 20 (Sigma), 0.5% bovine serum albumin (Sigma)). Then 20 μl of antibody solution was added containing 0.5 μg/ml mouse anti-BrdU antibody (1:1000 dilution

of stock, Pharmingen) and 1:2000 dilution of anti-mouse Ig antibody conjugated to HRP (Amersham) in PBSTB. The cells were incubated for 1 h at room temperature, washed twice with 90 µl PBS and then 20 µl HRP substrate solution was added to each well. The HRP substrate solution was obtained by mixing equal volumes of solutions 1 and 2 from the Amersham ECL detection kit. The plate was placed on a flat surface in a dark room, film (X-OMAT AR, Kodak Corporation) was placed on top of the plate for 1–5 min, and then developed in a Kodak M35A X-OMAT processor.

1536- and 6144-well anti-BrdU cytoblot

For 1536-well plates, 500 cells were seeded in 2 µl in each well of a white, opaque 1536-well plate (Corning/Costar) (cell density was 250,000 cells/ml). The cells were seeded in 1% medium containing the reagent of interest (e.g. 400 pM TGF-β1, Sigma). After 40 h at 37°C with 5% CO_2, 0.5 µl of BrdU was added, using a multichannel pipette, from a 5× stock in medium (prepared from a 1000× stock in PBS pH 7.4) to a final concentration of 10 µM BrdU. The cells were incubated for an additional 7 h at 37°C with 5% CO_2. See below for fixing and staining steps. For the 6144-well plate, 400 cells per well were seeded in excess 1% mink medium (cell density was 100,000 cells/ml). After 6.5 h, the media was changed to 1% mink medium with or without 400 pM TGF-β1 (Sigma) and the excess media was aspirated off, leaving 250 nl per well. After 18.5 h at 37°C with 5% CO_2, the medium was washed out and fresh 1% mink medium containing 10 µM BrdU was added. The cells were incubated for an additional 1.5 h at 37°C with 5% CO_2. For either 1536-well or 6144-well plates, the plate was cooled on ice for 15 min and kept under aluminum foil to minimize light exposure for all remaining operations. The plate was immersed in PBS and excess PBS was poured off. The cells were fixed by immersing the plate in a cold (4°C) solution of 70% ethanol/30% PBS and incubating for 1 h on ice. The plate was washed with cold (4°C) PBS and then 2 M HCl / 0.5% Tween-20 / ddH2O was added. The plate was incubated at room temperature for 20 min. The cells were washed once with a solution of 10% 2 M NaOH / 90% HBSS (GibcoBRL), twice with HBSS and once with PBSTB. Then antibody solution was added by layering onto the plate surface. The plate was incubated for 1 h at room temperature, washed twice with PBS and then HRP substrate solution was added. The plate was incubated for 1 min at room temperature and then wrapped in saran wrap and laid face down on a piece of film (X-OMAT AR, Kodak Corporation). Exposures of 1 min and 5 min were sufficient for detecting BrdU activity in mink lung cells. The film was developed in a Kodak M35A X-OMAT processor.

384-well acetylated histone H4, phospho histone H3 and phosphonucleolin cytoblots

Cells were seeded in 40–45 µl at the indicated cell density (typically 4000 cells/well), allowed to attach overnight (12–14 h) and then a known biological agent (e.g. trapoxin, 100 nM in DMSO for anti-acetylated histone H4 or 250 nM to 500 nM nocodazole for anti-phosphonucleolin) was added. After 4–24 h, the supernatant was removed from each well with a 24-channel wand attached to a vacuum source. 50 µl of cold (4°C) Tris-buffered saline (TBS, 10 mM Tris, pH 7.4, 0.15 M NaCl) was added to each well. The TBS was aspirated off and 40 µl of a cold (4°C) fixing solution of 3.7% formaldehyde in TBS was added to each well. The plates were incubated for 1 h at 4°C. The fixing solution was aspirated off and 30 µl of cold (−20°C) 100% methanol was added to each well. The plates were incubated at 4% C for 5 min. The methanol was aspirated off and each well was washed with 90 µl of 3% milk in TBS, then 25 µl of an antibody solution was added. Antibody solution contained appropriately either: 1:100 dilution of anti-acetylated H4 antibody (Upstate Biotechnology, Lake Placid, NY catalog number 06599) and 1:1000 dilution of anti-rabbit Ig antibody conjugated to HRP (Amersham) in 3% milk/TBS, a 1:100 dilution of anti-phospho Histone H3 Mitosis Marker antibody (Upstate Biotechnology, Lake Placid, NY, catalog number 06-570) and 1:500 dilution of anti-rabbit Ig antibody conjugated to HRP (Amersham) in 3% milk/TBS, or a 1:250 dilution of TG-3 monoclonal supernatant and 1:7500 dilution of anti-mouse IgM antibody conjugated to HRP (Calbiochem catalog number 401225). The plates were incubated for 2–24 h at 4°C. The antibody solution was aspirated off and the plates were washed twice with 90 µl of TBS. 30 µl HRP substrate solution was added to each well. The plates were allowed to incubate for 5 min at room temperature. The plate was placed on a flat surface in a dark room and a piece of film (Kodak X-OMAT AR) was placed on top of the plate. Exposures of 5–10 min were sufficient for detecting acetylation of histone H4 in A549 cells and 1–3 min were sufficient for detecting phosphorylation of histone H3 or nucleolin.

6144-well TG-3 cytoblot

For the 6144-well plate (Randy King, personal communication), cells were seeded on a 1 cm disk from an actual plate in a 24-well dish plate so as to have a final density of 300–500 cells per well by flooding the surface of the plate with cells in media. Cells were cultured overnight at 37°C with 5% CO_2. Media to a final volume of 1 ml was added and cells were either untreated or treated with 250 nM nocodazole for a 4–24 h period. Cells were washed and fixed as for above, except that solutions flooded onto the plate were aspirated off directly. Antibody solutions were added by layering onto the plate surface and incubated overnight at 4°C. The plates were then washed at room temperature overnight in TBS, the HRP substrate solution added by flooding the plate, and the plate was wrapped in saran wrap and laid face down on a piece of film.

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