Phenotypic Screening of Small Molecule Libraries by High Throughput Cell Imaging

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Abstract: We have developed high throughput fluorescence cell imaging methods to screen chemical libraries for compounds with effects on diverse aspects of cell physiology. We describe screens for compounds that arrest cells in mitosis, that block cell migration, and that block the secretory pathway. Each of these screens yielded specific inhibitors for research use, and the mitosis screen identified Eg5 as a potential target protein for cancer chemotherapy. Cell imaging provides a large amount of information from primary screening data that can be used to distinguish compounds with different effects on cells, and together with automated analysis, to quantitate compound effects.

INTRODUCTION

The Harvard Institute for Chemistry and Cell Biology (ICCB) was founded in 1997 to develop synthetic organic chemistry as a discovery tool in basic cell biology. Our approach is to synthesize small molecule libraries, screening them to find molecules with interesting biological activities, and use of these molecules to probe biological systems. Along the way we expect to discover target protein- ligand pairs that will catalyze drug discovery by industry, and to develop technology with broad applicability in drug discovery.

Most of the high throughput screens run at ICCB (>100 screens to date) have been phenotype-based. Phenotypic screens seek compounds with a specific effect on cell physiology, by adding compounds to living cells, and scoring for an effect. We have developed two major approaches to scoring specific physiological changes in cells in high throughput formats, "cytoblot" assays, and high throughput cell imaging. The cytoblot approach makes use of antibodies that recognize specific cell states, for example by binding to a phosphorylated protein epitope, and are read out with a typical plate reader [1]. Here we discuss high throughput cell imaging.

Cell imaging by fluorescence microscopy is one of the most widely used research tools in basic cell biology. Its power comes from the sensitivity and resolution of light microscopy, combined with the availability of fluorescent probes that allow specific imaging of essentially any cell component. These probes include antibodies labeled with fluorochromes, small molecules that bind directly to specific macromolecules and organelles, and protein fusions to green fluorescent protein (GFP), that allow, in principle, any cell protein to be expressed in fluorescent form. With these probes we can determine both the amount of a cell component, and most critically, its distribution within the cell relative to other components. Typically, 3-4 different components are localized in the same cell using probes that excite at different wavelengths. Any change in cell physiology should, in principle, cause a redistribution of one or more cell components, and this redistribution provides a diagnostic marker that allows scoring of the physiological change. Despite its power, and widespread use in basic research, fluorescence cell imaging has so far seen relatively little used as a readout in high thoughput screens. This may be changing as instruments and methods become more available [2]. Our goal in the work described here was to explore and develop the approach of screening by cell imaging, and at the same time, to find useful molecules for our research projects.

CHEMICAL LIBRARIES

Two sources of small molecules have provided the feedstock for ICCB screening projects. We synthesize our own libraries of structurally complex molecules using the principles of diversity oriented synthesis (DOS) [3]. We make DOS libraries by encoded, split-pool synthesis using a one-bead, one stock solution platform that delivers ~100 nmol of each compound in >75% pure form in 384-well stock plates [4,5]. This amount is sufficient for >100 screening assays in 384 well plates in either cell based or pure protein assays, including the methods described below. We have also developed a small molecule microarray method for detecting protein ligands, that allows >1000 screening assays per 100nmol of compound [6]. We supplement our DOS libraries with commercial collections of "drug-like" compounds from standard vendors. At the time of writing (3/03) we have ~200,000 compounds ready for screening, and we anticipate this number will grow rapidly as we expand our DOS libraries. Most of the work in this article used commercial collections, that became available for screening earlier than the DOS libraries.

EQUIPMENT

The key tools for high throughput cell imaging are suitable microtitre plates, and an automated fluorescence microscope. All our imaging screens have made use of standard commercial 384 well plates with black plastic wells and bottoms made of clear, thin plastic (Corning, Costar).

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We could obtain higher quality images by using microtitre plates with thin glass bottoms, but those are at least 20-fold more expensive, and fragile. Using 4x to 20x objective lenses, we obtain high quality images with the cheap plastic plates, and have found them suitable for culturing a wide variety of cell types. 384 well format was chosen as an optimal balance between minimizing the amount of library molecule required per assay, imaging sufficient cells per data point to generate valid statistics, and avoiding special engineering requirements and evaporation problems. We typically fill the wells with ~5,000 cells in ~40 μl of culture medium during cell plating and screening, and add library molecules by pin transfer. Using pins that transfer ~40-200 nl of stock solution, we achieve final compounds concentrations of ~10-50 μM in the screen.

Several vendors make automated fluorescent microscopes for screening applications. Our project called for a relatively simple, reliable instrument that enabled widefield imaging (as opposed to confocal) in at least 4 wavelength channels. We also required compatibility with industry standard plate handling robotics, and critically, flexible software tools for extracting quantitative measurements of physiological changes from fluorescent images. We have met these criteria at reasonable cost using two generations of automated microscope made by Universal Imaging Co. The first was a standard inverted microscope with added automation for plate positioning, autofocus and wavelength control, the second a purpose-designed instrument, the "Discovery-1" system (www.image1.com/products/discovery/). A key issue in automated microscopy is focussing. To date our instruments have used fluorescence imaging of the brightest signal in the experiment (typically a blue-fluorescing DNA dye as in figure 1) to find the correct focal plane by iterative cycles of focus travel, image capture, and calculation of image sharpness. The current instruments take ~10 images over ~2 seconds to find optimal focus, depending on how dissimilar the focal planes are in neighboring wells. One issue with the cheap plastic plates is non-flatness. The focal plane can change by >200 μm over the whole plate, requiring a focussing system with long travel. We anticipate moving to an interferometer-based method for focussing soon, which should speed it up ~10-fold. The microscopes use mercury or xenon arc light sources, and interference filters to control wavelength. A significant advantage of the Discovery-1 instrument is that it moves the dichroic mirror as well as the excitation and emission filters when changing wavelength, allowing for increased light throughput and shorter exposure times. Images are captured with a cooled CCD camera, stored, and analyzed off-line. Collecting images from each well of a 384 well in 3 wavelength channels currently takes ~90 minutes, depending on the brightness of the signals, and the number of images collected per well. This translates into a throughput of ~5,000 well per day on one instrument with modest automation, or ~10 days for a typical library screen. An interesting issue we are exploring in automated cell imaging is the optimal magnification for different experiments. We can gain surprisingly detailed information on cell

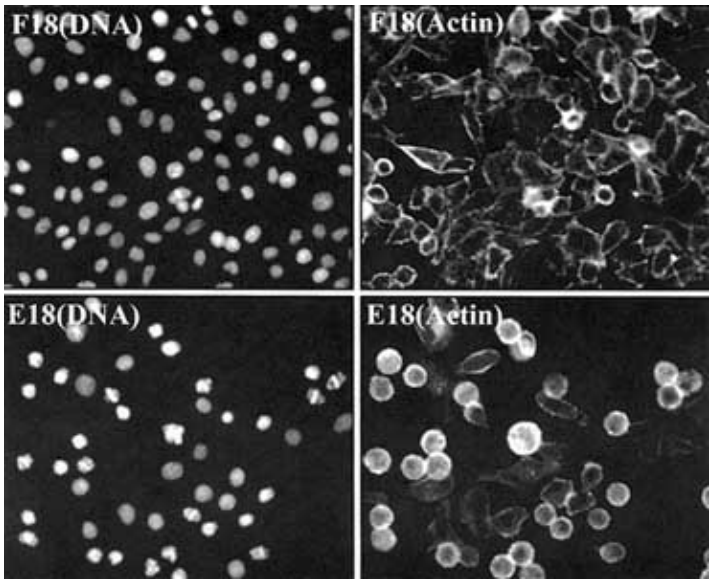

Fig. (1). Images from a screen for compounds that block cells in mitosis. HeLa (human) cells were synchonized by release from thymidine block, treated with compounds for 16hrs, fixed with formaldehyde, stained with DAPI and rhodamine-phalloidin, and imaged with a 10x objective. Well F18 is normal. In the DNA image most of the cells display a round interphase nucleus. The few mitotic cells display a normal metaphase plate or anaphase chromosome distribution. In the actin image most of the cells are well spread. Well E18 is a screening positive, with many of the cells arrested in mitosis. In the DNA image they display condensed chromosomes, distributed abnormally within the cell. In the actin channel the mitotic cells are rounded up. We do not know the target of the compound in well E18, but the DNA morphology is typical of a weak-moderate microtubule depolymerizer [8]. These images show only a fraction of the full field collected by the automatic microscope. From: N. Tolliday, J. Hoyt and T. J. Mitchison, unpublished.

morphology at relatively low magnification, for example with a 10x or even a 4x objective.

SCREENS

To provide a flavor of the range of cell physiology we have probed by high throughput cell imaging, we will outline three recent screening projects. Our first example is a screen for compounds that arrest cells in mitosis. Compounds with this effect that target microtubules (taxol, vinca alkaloids) are widely used in cancer chemotherapy, but they are quite toxic to neurons. Drugs that target proteins uniquely involved in mitosis are expected to kill cancer cells, but with less toxicity to non-dividing cells such as neurons. The first iteration of our screen for mitotic arrest used a cytoblot assay, with an antibody that recognized a phosphoepitope specific for mitotic cells [7]. This screen identified monastrol, a drug that targets the mitotic kinesin Eg5, as well as many compounds that target tubulin [8]. We subsequently found that cell imaging provides a more sensitive and informative readout of mitotic arrest. When mammalian cells are arrested in mitosis, their DNA is present in condensed chromosomes, and they round up. This state can be easily recognized in high throughput imaging format using stains for DNA and actin (Figure **1**). Using this method we have screened >40,000 compounds, and identified a large number that arrest cells in mitosis. The hit rate in this screen varies considerably between libraries. Commercial libraries, that are rich in flat, aromatic compounds, give a hit rate of 1-4%, because many such compounds bind to tubulin and promote microtubule depolymerization [8]. In a screen of a DOS library of dihydropyran carboxamides [9] we found a much lower hit rate (~0.3%), and obtained at least one hit with an interesting, non-tubulin target.

A major benefit of the imaging approach is that we obtain a lot of mechanistic information from the primary screen. Compounds that target tubulin cause a characteristic disruption of chromosomes distribution, ranging from clustering of chromosomes at the spindle poles at low dose, to completely random organization at higher doses. Compounds with other targets cause other effects. For example, inhibitors of the mitotic kinesin Eg5 cause chromosomes to accumulate in a circular arrangement around a single microtubule aster [7]. Recently we have found compounds that cause accumulation of cells in mitosis with apparently normal metaphase spindles. We have yet to identify the targets of these hits, but we suspect they may target part of the cell cycle regulatory machinery that is required for transition from mitosis into G1. Thus we can distinguish at least 3 different mitotic arrest phenotypes in our primary screen by cells imaging, while the cytoblot assay only told us if cells were arrested in mitosis or not. Cell imaging also allows us to identify compounds that cause a range of other effects, indicative of toxicity by differing mechanisms. These include cell lysis, rounding up without mitotic arrest, cell cycle block in G2, abnormal nuclear morphology, and many others. In general, we have found that the information we obtain by cell imaging – detailed cytological phenotype of cells arrested in mitosis,

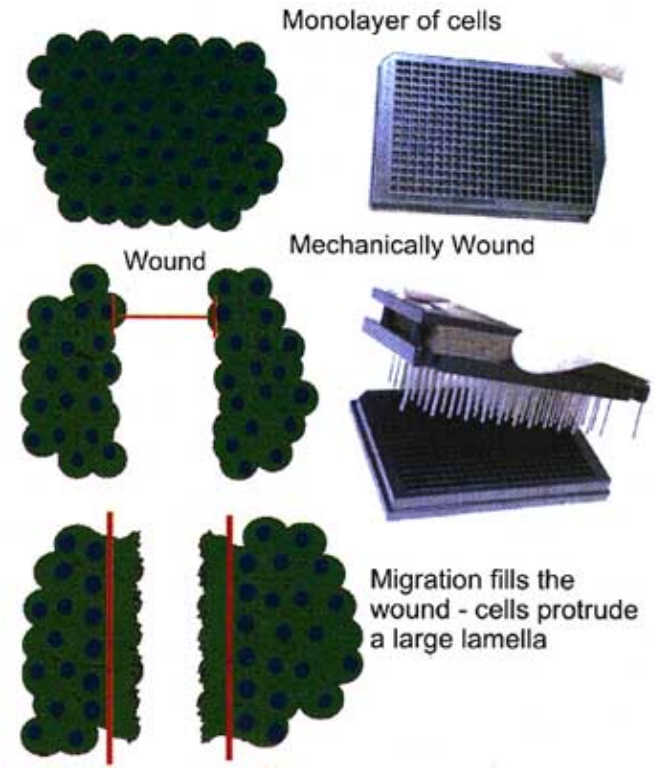

Fig. (2). Method for the wound-migration screen. A confluent monolayer of BS-C-1 (monkey) cells grown in 384-well imaging plates was scratched with a floating pin array to generate a wound. Cells bordering the vacant area migrate into it, in response to detecting a higher concentration of serum growth factors on their wound-proximal side. From: J. Yarrow and T. J. Mitchison, unpublished.

information relevant to toxicity by different mechanisms – justifies the slower throughput of cell imaging compared to plate reader screens.

Our second example, also in the cytoskeleton area, is a screen for compounds that perturb cell migration. Cell migration is important in many aspects of normal embryonic development and adult physiology, and it is implicated in diverse pathogenic processes including metastasis and angiogenesis during tumor progression. We sought compounds that perturb cell migration primarily for research use, with an eye to new approaches to cancer chemotherapy. The industry-standard method for screening compounds (or proteins) for effects on cell migration depends on trans-filter assays, where cells migrate from one part of a well to another through a filter with defined pores. 96-well plates for trans-filter migration assays are commercially available, but they are very expensive, and this method provides no information on cell morphology during migration. To provide a cheaper and more informative high-throughput assay, we adapted another standard method for measuring cell migration to high throughput, wound healing in tissue culture (Figures **2**,**3**). When a confluent monolayer of cells is scratched to produce a wound, cells at the wound margin experience a higher concentration of soluble trophic factors on the side facing the wound. If these factors stimulate cell migration, the cells migrate into the wound. Using different cell types and soluble factors, the wound-migration assay has been widely used in the basic science literature to measure cell migration. For our cell migration screen, we used a monkey epithelial cells line, and serum as a source of trophic factors. BS-C-1 monolayers were scratched with a floating pin array immediately prior to adding compound by pin transfer, and the plates were incubated for ~6 hrs to allow migration of cells into the resulting wound. After fixation and staining for DNA and actin, we imaged the wound area at low magnification. In control wells, migrating cells were easily recognized by their morphology, with a broad lamella and leading edge extending towards the wound (Figure **3**). Using cell imaging, we were able to score several morphological effects of interest. These included a block to migration, abnormal morphology of the leading lamella, toxicity (obvious damage to the whole cell monolayer, not shown) and mitotic block (cells rounded up with condensed DNA, see Figure **1**). We were most interested in the first two categories, and obtained ~300 compounds from ~16,000 screened in our first pass of a commercial library. Subsequent testing in 24-hr cell viability assays showed that the great majority of these screening positives in fact cause some type of generally toxic effect to the cell, that we consider less interesting. We are currently focussing on a single compound from the screen that blocks cell migration without causing general toxicity.

The cell migration screen was highly successful at a technical level. We were able to rapidly screen a library for compounds with the desired physiological effect, at a fraction of the cost of using trans-filter migration plates, and

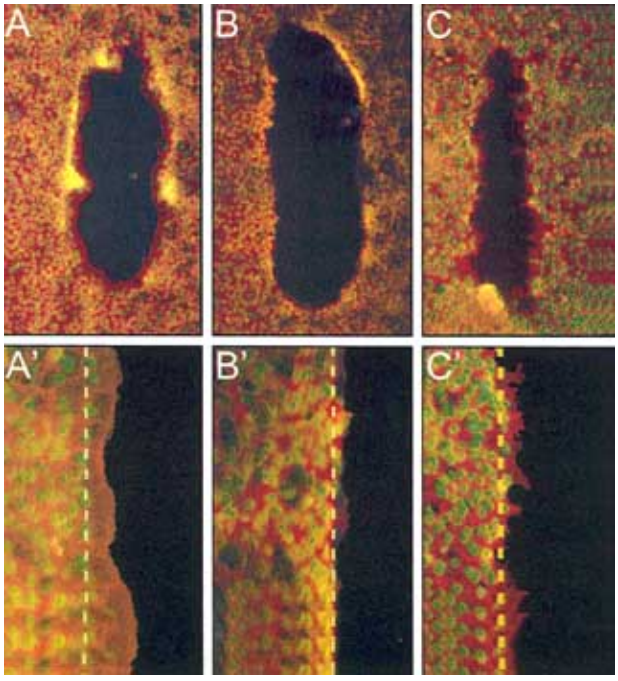

Fig. (3). Images from the wound-migration screen. Shown are images from control two phenotypes of interest. A,A'; control. Note the broad leading lamella extending into the wound. B,B'; migration blocked. Note the leading lamella is completely absent. C,C': abnormal morphology. Note that the cells can obviously detect the wound margin, and have begun to migrate, but the morphology of the leading lamella is perturbed, appearing spiky and disconnected from neighboring cells. A,B,C; images collected with a 4x objective lens from the actual screen. This magnification allows the whole wound to be viewed. A'B'C'; images collected with a 10x objective lens during re-testing of screening positives. This magnification allows detailed assessment of morphology. From: J. Yarrow and T. Mitchison, unpublished.

we obtained detailed morphological information relevant to the mechanism of compound action. The assay method should be readily adaptable to different cell types, and different factors in the culture medium that promote chemotaxis into the wound. Our method could thus be relevant to many different drug discovery projects. Using generic cells, multiple serum factors to promote migration, and a small commercial library, we found only a single compound that reversibly blocks cell migration without causing toxicity after long term treatment. Subsequent iterations of the project might be more effective in identifying useful compounds if they either included a counter screen for toxicity in the primary screen, or screened libraries of partially optimized compounds, or protein factors, so that generic toxicity was not an issue.

The last example is a screen for compounds that block the secretory pathway. Secreted and membrane proteins translocate into the endoplasmic reticulum (ER) during translation, where they fold and are glycosylated. Then they are transported to the cell surface via the Golgi apparatus by a complex vesicle trafficking pathway. The details of this pathway are still poorly understood, and we sought small molecule inhibitors as research tools. Our screen was based on a method for synchronizing exit from the ER, using a temperature sensitive mutant of a viral glycoprotein (ts-VSV-G) that accumulates in the ER at 40°, and then exits the ER in a synchronous wave when the cells are cooled to 32°. We fused ts-VSV-G to GFP, and expressed the fusion protein in BSC1 (monkey) cells using an adenovirus vector that allows uniform transfection and expression. Cells were grown at 40° overnight to accumulate the protein in the ER, then treated with compounds, and then either held at 40°, or shifted to 32°. At the high temperature the ts-VSV-G remains in the ER, and imaging allowed us to determine the effect of compounds on ER morphology. At the low temperature the ts-VSV-G exits the ER and moves through the Golgi to the cell surface. Imaging of these cells allowed us to determine the effect of compounds on post-ER steps in trafficking to the cell surface. Using this assay we were able to score 4 different phenotypic effects caused by compounds (Figure **4**), as well as more generic toxicity. Two of these phenotypes, block to exit from the ER, and block to exit from the Golgi, represent blocks at different steps in the secretory pathway, effects we hoped to identify in the screen.

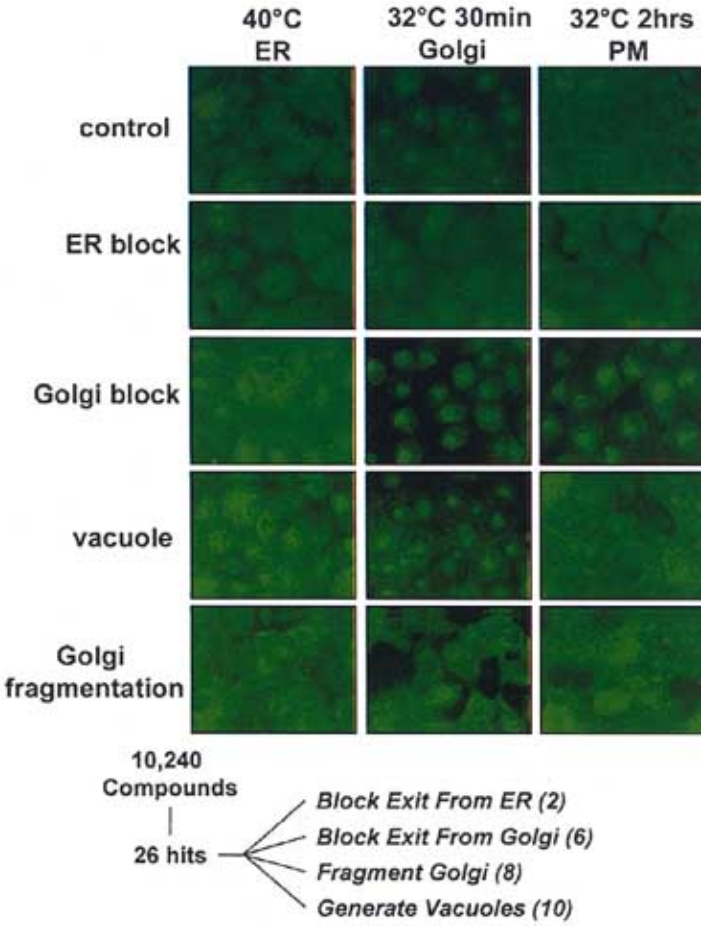

Fig. (4). Screen for compounds that block the secretory pathway. ts-VSV-G protein fused to GFP was expressed in BSC1 (monkey) cells using an adenovirus vector. Cells were incubated at 40° to accumulate the protein in the ER. Compounds were then added, and the cells were either kept at 40°, or shifted to 32°, incubated to allow compound action, then fixed and imaged. Shifting to 32° for 30' allowed most of the ts-VSV-G-GFP to reach the golgi, and 32° for 2hr allowed it to reach the plasma membrane. The images show control and 4 different phenotypes we scored. The number of screening positives scored in each category is shown below. From: Feng *et al.* [10].

The screen was performed on ~10,000 commercial compounds, yielding the hits shown in Figure **5**, and a DOS library of galanthamine-like compounds, that yielded an interesting hit, secramine (Pelish *et al.* 2001). One of the commercial compounds that blocks exit from the ER (6B6, or exo1) has a brefeldin-A like effect, that we have characterized in detail [10]. We have not yet extensively characterized the compounds that block exit from the Golgi. These, together with secramine, are the first known compounds with this effect on cells, and may provide useful research tools. The other two phenotypes we scored are not specific blocks to the secretory pathway, but rather represent other interesting perturbation of cell physiology that we were able to score by imaging our ts-VSV-G-GFP probe. Several related compounds potently cause large vacuoles to accumulate in the cytoplasm. The cells do not die, and remarkably, secretion still occurs. The endosomal vesicle-trafficking system is severely perturbed by these compounds, and we are now probing their mechanism in detail (Cerny, Feng and Kirchhausen unpublished). Several different compounds caused the golgi apparatus to fragment. By cross-referencing to the mitosis block screen, which was performed with the same compound library, we determined that most if not all of the golgi fragmentation compound also perturb mitosis. We infer that they most likely depolymerize microtubule by binding to tubulin, since microtubule targeting drugs are known to cause golgi fragmentation [11]. Indeed several of these compounds were shown to destabilize microtubules, and one, 12A11, has a taxol-like effect, stabilizing microtubules [8]. Overall, the secretion screen was successful in providing several new tools for research into vesicular trafficking, and it illustrates the power of cell imaging to reveal compounds with multiple mechanisms of action from a single screen.

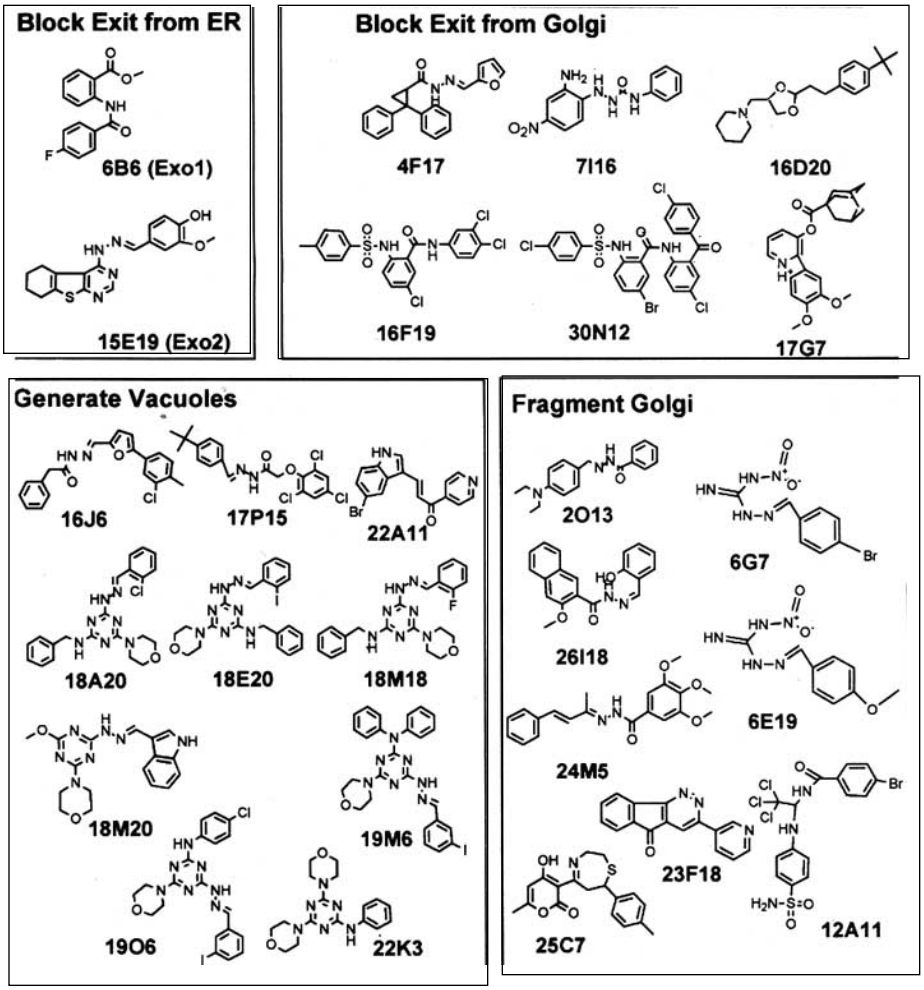

Fig. (5). Hits from the secretory pathway screen. The targets of these compounds are unknown, except for the golgi disrupting class, many of which target microtubules. From: Feng *et al.* [10].

DATA ANALYSIS

In each of the three projects described above, the images were scored by visual inspection of stored images. The human eye is very effective at detecting certain kinds of expected effects in images, and it is also very good at identifying and categorizing unexpected effects. However it is difficult to obtain reliable quantitative information simply by visual inspection, and tedium becomes a major issue once the number of images to be scored is >20,000. Thus we need reliable and flexible methods for extracting quantitative measurements of physiological changes from fluorescent images of cells. Our UIC software provides tools for this, and we are developing more ourselves. We have now developed semi-automated methods for scoring both the mitotic and cell migration screens, that flag wells of interest (typically <5% of the total) for subsequent confirmation by eye. Data analysis typically involves some type of thresholding algorithm to outline cells or internal organelles of interest, then measurement of the size, shape, relative position, or amount of fluorescence in the identified objects. This results in a histogram of measured parameters for each cell in the image (typically >200), that can be compared to histograms of the same parameter for control cells. Figure **6** shows an example where the distribution of chromosomes within mitotic cells is measured. The data are taken from a successful screen for compounds that overcome the action of monastrol. Note that the duplicate screening positive is well separated from the no effect wells by the analysis algorithm. We are currently working on developing software tools for measuring multiple parameters that are relevant for many different screens, and better methods for weeding out false positives due to low cell number, out of focus images etc. We anticipate that future cell imaging screens will use automated image analysis as their primary readout.

DISCUSSION AND FUTURE DIRECTIONS

High throughput cell imaging has proven an effective method for identifying compounds from chemical libraries with specific effects on a broad range of cell physiology. Compared to plate-reader methods that generate a single number for each compound, imaging provides much more

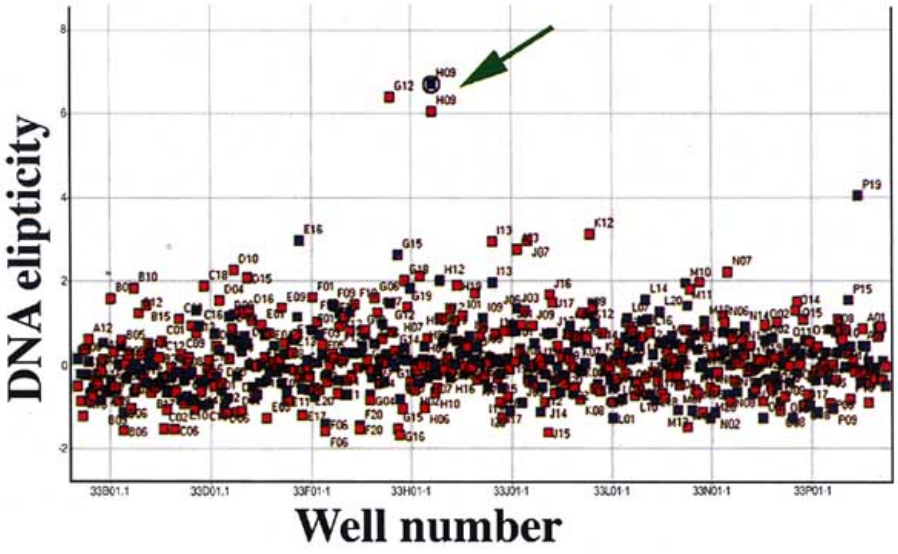

Fig. (6). Automated data analysis of a cell imaging screen. This graph shows one example of how we are starting to automate scoring of cell imaging screens, using image analysis. In this screen we sought compounds that reversed the effects of monastrol, an Eg5 inhibitor that arrests cells in mitosis with their chromosomes arranged in a circle. The images were generated as in Fig. (**1**). We used the actin image to identify mitotic cells by their rounded morphology, and then thresholded the DNA image to describe an ellipse that encompassed the chromosome distributions. We calculated the ratio of the long to the short axis of this ellipse (ellipticity) for each mitotic cell, and used the median value to read out the effect.. This median ellipticity parameter allowed us to reliably distinguish wells in which chromosomes were arranged in a circle in most mitotic cells from wells in which they were aligned in a bar-shaped metaphase plate in most cells. The plot shows the median ellipticity of the DNA distribution for one plate, scored in 2 duplicate runs. The duplicate wells are shown grey and black. Note that the median ellipticity is reproducibly low in most wells, indicating no effect of compound. In well H09 (arrow) a compound has caused a increase in ellipticity in both duplicates. This change is due to formation of normal metaphase plates, by reversing the effect of monastrol. The non-duplicate high value well (G12) was an artifact due to low cell number or out of focus image. This analysis method allowed us to efficiently find monastrol reversing compounds. From: Perlman, Mayer, and Mitchison, unpublished.

information, without much increase in cost per assay point. For our projects the large information content has justified the slower throughput of imaging. What are the current limitations, and where might this technology go in the future?

With respect to ICCB projects, we see several areas where we can make significant improvements in high throughput imaging in the short term. The most obvious is data analysis. We need flexible image analysis tools for rapidly extracting quantitative measurements of the effects we seek, and of information relevant to other aspects of compound action. These tools need to be integrated with primary image storage, and with our databases of chemical structures and activities. Implementation of such data analysis tools will make image based screening just as convenient, but significantly more powerful than, our plate-reader based phenotypic screening methods. Hardware improvements will also be useful, such as speeding up auto-focus by implementing laser based interferometry. We are currently not interested in screens on living cells, and collecting time-dependent information on each well, but this might be important for probing certain aspects of cell physiology in the future.

The most important bottleneck in our current project at present is in fact unrelated to screening per se, but rather represents the limiting step for the whole approach of phenotypic screening. To move from an interesting observation to a ligand/target pair we need to determine the molecular mechanism of compounds action, or identify its target protein. We refer to this challenge as the "target ID" problem, and are currently working on solving it in several ways, including affinity based methods, genetic selection for genes that modify the cell's response to a compound, and testing candidate proteins that are suggested by detailed analysis of the phenotype. Currently, we are tackling the target ID problem on an ad hoc, compound by compound basis, taking advantage of the chemistry of the compound, the biology of the cell system, and how much is already known about the physiological process of interest. In the future it would be better to develop a generals method for target ID. Target ID is an important future challenge both for us, and for the wider field of chemical biology.

One interesting future direction will be to extend image based screening to more cell types and phenotypes, including some more directly relevant to therapeutic drug discovery. We are interested, for example, in screens that use neurons in the wells, or screens that use more than one cell type to recapitulate in culture some interesting aspect of organ physiology. Another important path we have started down is to greatly increase the information we glean from cell imaging, by adding more probes, and measuring more features in the images. We plan to generate in this way a "quantitative cytological profile" of compound action, that describes the effect of a compound on a broad range of physiological processes in a computer-accessible form. This profile will look something like a gene expression profile obtained with DNA chips, and anticipate using it in ways that have already been explored for gene expression profiles, for example, to cluster compounds with related mechanisms of action. Such clustering should allow us to discover new compounds with an effect similar to a known compound. It will also suggest likely targets for a compound of unknown mechanism, and help us understand side-effects of therapeutic drugs. Cytological profiling is in principle orthogonal to profiling by systematic measurement of gene expression or protein modification. Combining these analysis methods will generate a more complete description of phenotype compared to any single analysis. This multi-parameter, quantitiative phenotype, that embraces both gene expression and cell organization, should be of considerable value in both drug discovery and basic research.

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