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Exploiting Network Biology to Improve Drug Discovery

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Summary

Mammalian signal transduction occurs in the context of multiprotein complexes, yet currently available drug discovery strategies do not reflect this fact. We present a strategy for screening drugs and targets in living human cells by utilizing high content protein-fragment complementation assays. Synthetic fragments of a mutant fluorescent protein (“Venus” and/or enhanced yellow fluorescent protein) are used for protein-fragment complementation assay construction, allowing us to measure spatial and temporal changes in protein complexes in response to drugs that activate or inhibit particular pathways. Here we describe the utility of this novel strategy for high-throughput screening of known targets, and for screening previously undruggable targets and profiling drug leads for improved selectivity and safety.

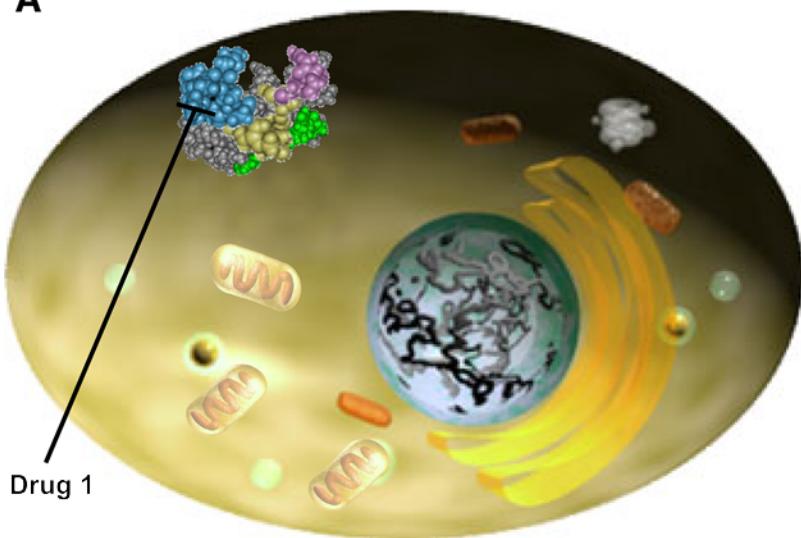
Key Words: Cell-based assay; chemical biology; drug discovery; fluorescence microscopy; G protein-coupled receptor; high content screening; kinase; network; nuclear receptor; pathway; protein complexes; protein-fragment complementation assay; signal transduction; transcription factor.

1. Introduction

The modern drug discovery paradigm involves selecting a target, establishing an *in vitro* assay, and then identifying a molecule that binds to or regulates the target. This is accomplished by high-throughput screening of a chemical library and/or by rational design of a compound based on crystal structure of the target-binding site. Current approaches treat the cell as a black box, and cell-based methods are usually limited to secondary assays, which are designed primarily to verify the bioavailability and potency of the lead compound. However, an isolated protein in a test tube is far removed from its native cellular and subcellular context, and from the many other molecules that influence its structure, activity, and ultimate function. Cells are complex systems in which a multitude of biochemical reactions take place at any one time. Protein targets of drugs often exist as components of dynamic multiprotein complexes, connected to many other proteins and macromolecules. Further, the same protein often exists in different forms, as components of different complexes and in different cellular compartments (**Fig. 1**). An example of this phenomenon is found in the Akt signaling pathway. Akt is found primarily at the plasma membrane, but also exists in the nucleus. We have observed that PI3K-phosphoinositide-dependent kinase (PDK)/Akt complexes at the plasma membrane are regulated by drugs (such as PI3 kinase inhibitors), whereas nuclear complexes containing Akt and p27 are regulated by other drugs (schematized in **Fig. 1**). Also, even a highly potent, targeted compound might regulate the activity of dozens of proteins apart from its intended target or even its target class. Thus, identifying the full spectrum of activity of any drug or drug candidate is not possible with *in vitro* assays, regardless of the size of the panel. We

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A Drugs act on multiprotein complexes in living cells



B

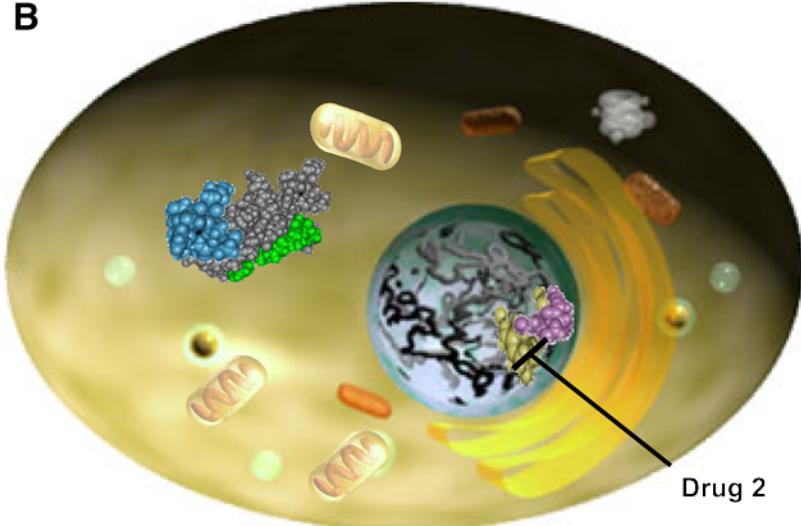


Fig. 1. Proteins in cells exist as large, multimolecular structures, and drugs act in the context of these complexes. A representation of a protein complex is shown (A). Drug 1 acts on a protein complex located in the plasma membrane. In (B), a portion of the complex is localized in the nucleus. In this case, drug two regulates the nuclear protein, but drug one is not active in this context.

believe that the living human cell is the central, missing component in preclinical drug discovery, and that both potential drug liabilities as well as beneficial applications might be lost because of the unforeseen actions of drugs within the cellular milieu.

The need to examine global effects of drugs on human cells has inspired the applications of DNA microarrays, including the field of toxicogenomics, which involves the use of complex populations of mRNA to understand toxicity. Cells, or whole animals, are treated with drugs; messenger RNA is isolated from the cell or tissue; and the gene expression patterns of the mRNA in the absence and presence of a drug are compared. Such transcriptional profiling can identify subtle differences between compounds, in which the compounds affect the ultimate transcriptional

activity of one or more pathways (1). Identifying specific genes that are stimulated or repressed in response to specific conditions or treatments is a useful way to begin to unravel the cellular mechanisms of disease and of drug response. However, changes in the level of individual mRNA molecules correlate poorly with the level of the corresponding protein, and even less with protein activity (2). Furthermore, many proteins undergo numerous posttranslational modifications and protein interactions, which might affect the functions and activities of proteins within a tissue or cell. Thus, simply measuring the mRNA species present at a particular time does not yield an informative picture of a drug. Finally, a targeted drug might affect the transcription of dozens or hundreds of genes, making interpretation of drug mechanism of action difficult to deconvolute (3).

Direct temporal and spatial measurements of specific chemical transitions within biochemical pathways could eliminate the problems associated with interpretation of drug perturbations of transcriptional profiles. Among the most obvious and general transitions that could be measured are the induction and dissociations of protein complexes. Unlike transcriptional reporter assays, the information obtained by monitoring protein complexes reflects the effect of a drug on a particular branch or node of a biochemical pathway, not its end point (4,5). For example, stimulation of a pathway by an agonist could lead to an increase in the association of an intracellular protein (such as a kinase) with a cognate binding partner (such as a substrate). The drug effect could therefore be assessed by quantifying the amount or location of the kinase/substrate complex in the absence and presence of the drug. In this example the kinase/substrate complex serves as a “sentinel” of pathway activity. A drug acting either at the beginning of the pathway (such as a receptor antagonist) or acting on another target downstream of the receptor but upstream of the sentinel could alter either the amount or location of the kinase/substrate complex within the cell. Thus, assessing complexes in the absence or presence of a chemical compound could reveal whether or not the drug acts on that pathway.

In addition to being composed of large multiprotein complexes, biochemical pathways are highly organized in subcellular space. For instance, in signal transduction pathways, membrane receptors that receive signals at the plasma membrane transmit the signals to the cell nucleus, resulting in the activation or repression of gene transcription. Thus, the subcellular compartment of a particular complex reflects the function of that complex at a particular point in time (see Figs. 1–3D). High content methods that allow for the visualization and quantification of protein complexes in subcellular compartments can provide far more information than simple bulk measurements of fluorescence.

In this chapter we review high content assays for studying the spatial and temporal dynamics of protein complexes based on protein-fragment complementation assays (PCA) and provide examples of their applications to drug discovery.

2. Localization and Quantification of Protein Complexes in Cells

A protein–protein interaction assay is capable of measuring either the interaction between two proteins (in network biology terms, the “transition”) or the complex that is formed as a result of an interaction (the “state” of the proteins that results from the interaction). PCA represents a particularly useful strategy for the measurement of protein complexes in living cells. At its basic level, PCA is a universal and flexible strategy that allows for the detection, quantification, and localization of protein–protein complexes in intact, living cells.

Michnick and coworkers (see ref. 6 herein) first showed that a reporter protein can be intentionally fragmented in such a way that the two polypeptide fragments, when fused separately to two other molecules that interact, are capable of refolding and reconstituting the activity of the original reporter. Because all of the information for protein folding is encoded in the primary amino acid sequence of the protein, close apposition of the fragments—as a result of the interaction of the molecules to which the fragments are fused—is sufficient to cause the folding of fragments into an active protein, thereby recapitulating the unimolecular folding reaction of the

Proteins function as complexes in particular subcellular compartments related to their functions

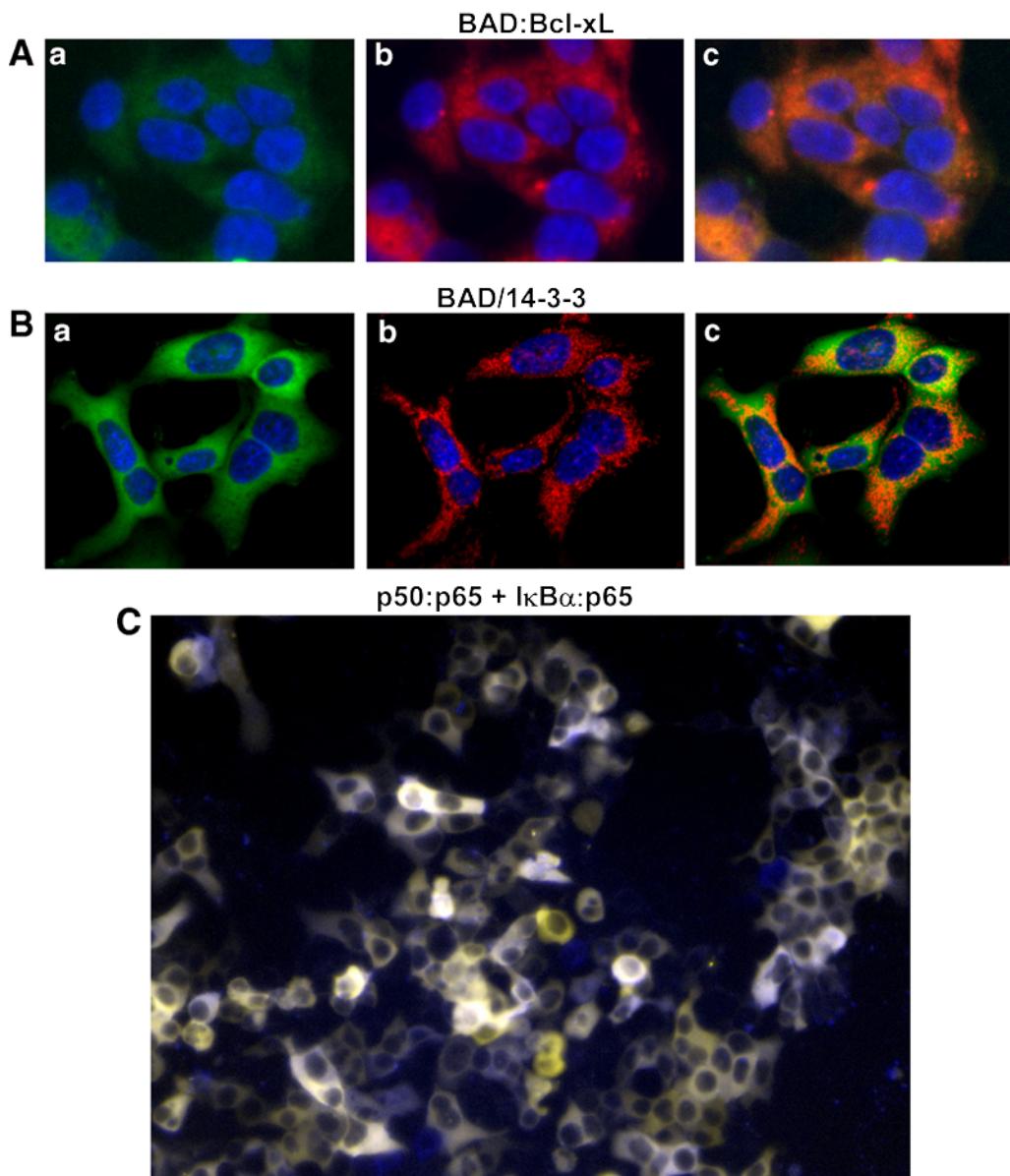


Fig. 2. (A,B) BAD complexes in cells as assessed with a fluorescence (enhanced yellow fluorescent protein) PCA. (A) BAD/Bcl-XL; (B), BAD/14-3-3-sigma. Shown are: (a) the PCA signal (green; nuclei are stained with Hoechst), (b) a mitochondrial dye (Mitotracker red, Molecular Probes), and (c) overlay of (a) and (b). (C) Dual-color PCAs for the NF κ B pathway, showing I κ B α /p65 (yellow fluorescence) and p50/p65 (blue fluorescence). Cells were transiently transfected with the three fragment constructs. Different colors are generated according to the interactions of the proteins to which the respective fragments are fused. Fluorescence images were acquired with an SP Nikon fluorescence microscope using a Chroma CFP filter (excitation: 426–446 nm; emission: 460–500 nm; dichroic mirror: 455LP), and a FITC filter (excitation: 460–500 nm; emission: 505–560 nm; dichroic mirror: 505LP). 16-bit monochrome images were acquired with a CoolSnap HQ CCD camera. CFP and FITC images for each PCA were subsequently pseudo-colored and overlaid using Metamorph software (Molecular Devices, Sunnyvale, CA).

Types of dynamic changes that occur in response to drugs or siRNAs

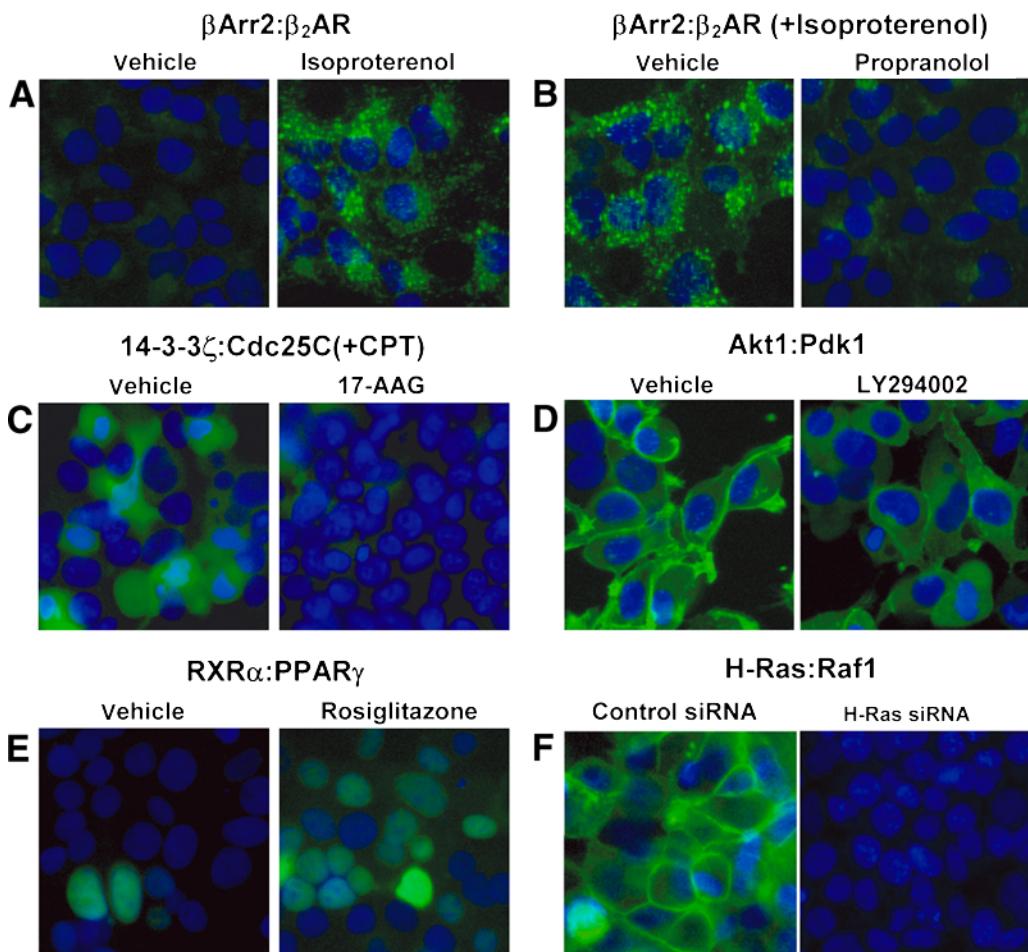


Fig. 3. Examples of dynamic changes that occur in response to drugs or siRNAs include increases, decreases, or change in localization of the fluorescence signal. PCAs and pretreatment conditions, in which used, are as follows: (A): β -arrestin2/ β_2 -adrenergic receptor; (B) β -arrestin2/ β_2 -adrenergic receptor (+isoproterenol); (C) 14-3-3- ζ /Cdc25C (+CPT); (D) AKT1/PDK1; (E) RXR α /PPAR γ ; (F) H-Ras/Raf1.

original reporter and the reconstitution of enzymatic activity. Subsequent studies suggested that, if a flexible linker of 10 amino acids is included between the interacting protein(s) of interest and the complementary fragments, a distance of 80 Å or less allowed refolding of the reporter fragments and reconstitution of reporter activity. Thus, protein-fragment complementation serves as a direct assay of complex formation between molecules to which the fragments are fused. PCAs based on nearly 100 different enzymes and fluorescent proteins have been conceived of, and many of these have been constructed, including GFP and a number of mutant fluorescent proteins (cyan, yellow, citrine, red fluorescent proteins, and variants thereof), luciferases from various species, dihydrofolate reductase, β -lactamase, hygromycin phosphotransferase, GAR transformylase, and others (6–15). Recently, we have constructed PCAs based on intense and rapidly maturing variants of yellow fluorescent protein (YFP) to probe hundreds of different known and novel protein complexes, which include all major classes of drug targets and biochemical pathways, different

subcellular compartments, and pathways that are both general and specific to differentiated cell types. In this review, we discuss examples taken largely from signal transduction pathways.

Since the original invention in 1997, the PCA technology has sometimes been dubbed with other names, including interaction-dependent enzyme activation, bimolecular fluorescence complementation, trimolecular fluorescence complementation, “half-zyme,” “split-GFP,” “split-luciferase,” and “luciferase complementation imaging,” all of, which refer to the same basic technology (16–20). Owing to the diversity of reporter proteins that can be used to construct PCAs, assays can be tailored to the particular demands of the cell type, target, signaling process, affinity of the binding partners, and screening instrumentation. Finally, the ability to choose among a wide range of reporter fragments generating fluorescence signals provides a choice of high-content or high-throughput assay formats.

PCA has unique features that make it an important tool in drug discovery:

- The PCA strategy is applicable to any cell into which DNA constructs or their encoded protein products can be introduced, including prokaryotic and eukaryotic cells and primary cells.
- Molecular interactions are detected directly, not through secondary events such as transcriptional activation or second messenger production.
- Tagging of proteins with large molecules, such as intact fluorescent proteins, is not required. In contrast to assays utilizing intact reporters, many PCAs reveal dramatic drug- or agonist-induced changes in total fluorescence, not just changes in subcellular localization (see, e.g., **Figs. 3** and **4**).
- Proteins are expressed in the relevant cellular context, reflecting the native state of the proteins with their correct post-translational modifications and in the presence of other enzymatic and structural components that regulate the complexes.
- PCA fragments can be synthesized and/or genetically engineered to create assays with specific properties (signal intensity, stability, spectral properties, wavelength, and so on).
- Flexibility in expression vector design enables the choice of various gene orientations, linker lengths, constitutive or inducible promoters, plasmid or viral vectors, and various selectable marker strategies depending on the assay demands.
- PCA can be used to map proteins and drugs into signaling pathways and validate novel targets by determining whether the protein–protein complex(es) can be modulated in response to an agonist, antagonist, inhibitor, or siRNA.

3. Measuring Distinct Complexes Formed in Different Specific Subcellular Locations

Considering that all proteins exist in complexes, and interact with multiple proteins, it is not surprising that any particular protein might function simultaneously or sequentially in different subcellular compartments in conjunction with different interacting partners. These relationships are usually inferred from colocalization studies, but such studies only show that two proteins are localized in the same compartment at the same time—not that they exist in a physical complex. Direct analysis of these interactions can be achieved with high content PCAs. **Figure 2(A,B)** shows two different protein complexes, both of which involve the protein Bad. Bad is a member of the BH3-only subfamily of apoptosis-regulating proteins. When Bad is phosphorylated it is found in the cytosol, where it is bound to 14-3-3 proteins and where it is incapable of inducing cell death. Protein 14-3-3 is a family of multifunctional phosphoserine peptide-binding molecules that serve as effectors of survival signaling. BAD is known to form complexes with the protein 14-3-3 as well as with other members of the BAD family such as Bcl-xL. As shown in **Fig. 2A**, Bad/Bcl-xL complexes are located in the mitochondria, as the PCA signal (green) is colocalized with the mitochondrial dye (red). In contrast, Bad/14-3-3 complexes are clearly located throughout the cytosol of the cells (**Fig. 2B**). Importantly, the different BAD complexes carry out different, highly specialized, functions in the cell. Differentiation of these functions requires analysis of specific BAD complexes, and can only be studied at the subcellular level using high content methods.

Dose-dependent effects of drugs on protein interactions, as assessed by automated microscopy for Chk1:Cdc25C

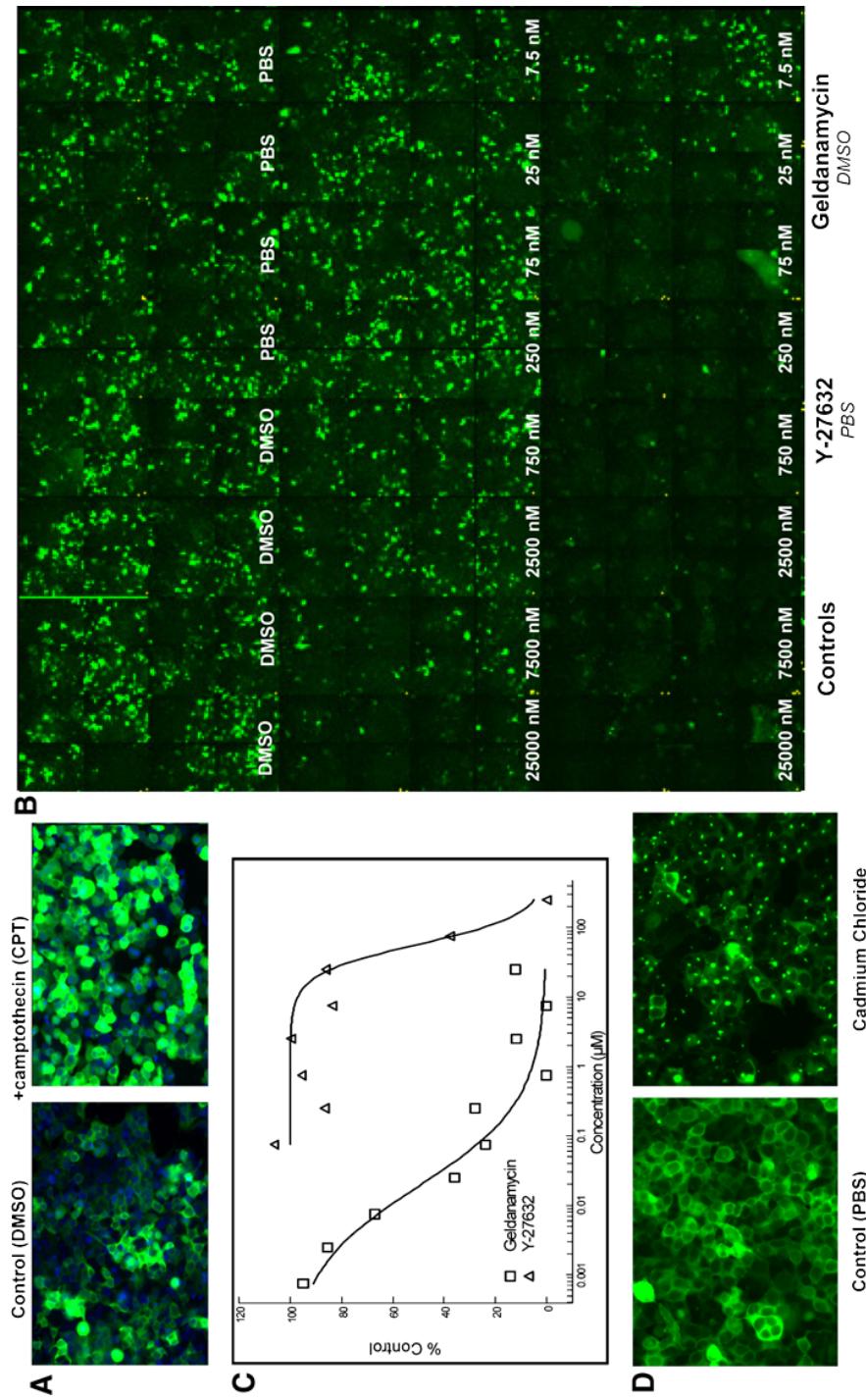


Fig. 4. Dose-dependent effects of drugs on protein interactions, as assessed by automated microscopy for Chk1/Cdc25C in HEK293 cells stably expressing the two constructs. **(A)** CPT induces the interaction of Chk1 with Cdc25C in the cell nucleus. **(B)** Y27632 and geldanamycin suppress Chk1/Cdc25C complexes in a dose-dependent manner, as seen in this 96-well plate view which shows eight different scans for each drug dose. **(C)** Dose-response curves for the drugs based on total fluorescence. **(D)** High content changes in signal pattern showing the formation of bright subnuclear foci in response to cadmium chloride.

We see similar compartmentalization with dozens of other well-characterized protein complexes. This means that simple tagging of a protein with a fluorophore such as GFP—which is done to monitor translocation of individual proteins or colocalization of two different proteins—only illuminates the bulk of the tagged protein in the cell, and completely ignores the specialized functions of proteins in the context of their subcellular compartments and interacting partners. Drug effects might be missed if the bulk changes in individual proteins are studied, but can be readily detected if dynamic protein complexes are monitored. Moreover, monitoring of different complexes in the same pathway allows for the determination of the mechanism of action of a drug at different steps, allowing for the site of action of the drug to be narrowed down to a range of, or to a specific, protein. **Figure 2C** provides an example of multiplexing this type of analysis with multicolor PCA in living cells. The NF κ B p65 subunit forms complexes with the p50 subunit, and also with the protein I κ B α . In resting cells, I κ B α binds to NF κ B and retains the complex in the cytoplasm. Thus, p65 forms cytoplasmic protein–protein complexes with p50 and also with I κ B α . We cotransfected HEK293 cells simultaneously with three PCA constructs: CFP[1]-p50; CFP[2]-p65; and I κ B α -YFP[1]. If a protein–protein complex forms between p50 and p65, the CFP[1] fragment should complement the CFP[2] fragment, producing blue fluorescence. Alternatively, if a protein–protein complex forms between I κ B α and p65, the YFP[1] fragment should complement the CFP[2] fragment, producing a yellow fluorescence. As shown in **Fig. 2C**, both p65/p50 (blue) and I κ B α /p65 (yellow) complexes could be detected in the cytoplasm as expected. Cells displaying a lighter yellow (almost white) cytoplasmic signal are expressing both p65/p50 and I κ B α /p65 complexes. The ability to construct multicolor PCAs allows for the detection and quantification of multiple distinct protein–protein complexes within the same cells.

4. Measuring the Dynamics of Protein Complexes With High-Content PCAs

A key feature of PCA, as compared with *in vitro*, proteomics-based approaches, is the ability to capture the *dynamics* of complexes in their native context. In conjunction with high content cell imaging, detailed information on the levels and localization of signaling complexes can be obtained. Thus, PCA can be used not only to identify the components of signaling networks, but also to probe the activity or flux of pathways within these networks. The examples below demonstrate that it is possible to assay discrete signaling nodes for agents that act directly on the signaling proteins of the interaction, as well as for targets and agents that act “upstream” of the interaction. The strategy works equally well for assessing effects of small molecules or genetic probes such as dominant negative/active clones and RNAi on signal transduction pathways.

Six different examples of dynamic interactions are shown in **Fig. 3**. The examples include a GPCR/GPCR modulator interaction (**A,B**); a cell cycle phosphatase/regulatory protein interaction (**C**); a kinase/kinase interaction (**D**); a nuclear hormone receptor/coactivator interaction (**E**); and a small GTPase/kinase complex (**F**). β -Arrestins are regulatory proteins that form complexes with most GPCRs and play a central role in receptor desensitization, sequestration, and down-regulation (for a review see Luttrell and Lefkowitz [21]). β -arrestin binding to GPCRs both uncouples receptors from their cognate G proteins and targets the receptors to clathrin-coated pits for endocytosis. β -arrestins might also function as GPCR signal transducers. β -Arrestin movement from the plasma membrane to intracellular vesicles has been visualized by tagging β -arrestin with GFP and monitoring the subcellular distribution of the fluorescence signal in living cells (22). We sought to create a quantitative fluorescence assay for which changes in GPCR activation would be detected by an increase or decrease in the reconstituted fluorescent signal generated by binding of the receptor to β -arrestin. A stable HEK293 cell line expressing the constructs β -arrestin2-IFP(1) and β 2AR-YFP(2) was generated. Cells were treated at 37°C with 10 μ M isoproterenol for 60 min. Cells were fixed and stained and images of the reconstituted fluorescent signal were acquired on a Discovery-1 instrument (Molecular Devices, Sunnyvale, CA)

using a $\times 20$ objective. **Figure 3A** demonstrates the induction of fluorescence in response to agonist (isoproterenol) and the appearance of signal in intracellular granules. The appearance of the protein–protein complex in intracellular granules (corresponding to endosomes) is consistent with the process of receptor internalization in response to agonist, and the effect can be blocked by pretreatment of cells with the reverse agonist, propranolol (**Fig. 3B**). It is important to note the key distinctions between this PCA and previous cell-based assays involving a GFP-tagged β -arrestin, which measures a different phenomenon. Direct tagging of a protein, such as β -arrestin, with a fluorescent protein or other optically detectable molecule only enables imaging of its subcellular localization (22). In contrast, PCA quantifies the association of two proteins and the subcellular location of the protein–protein complex. Other examples of dynamic changes of protein complexes are shown in **Fig. 3**. **Figure 3C** shows a complex between a 14-3-3 protein and Cdc25C. These complexes are induced by camptothecin (CPT). Treatment of the cells with an HSP90 inhibitor such as 17-AAG (17-allylaminogeldanamycin) causes destruction of the complex, because of ubiquitin-mediated proteolysis of PCA-components that are client proteins of HSP90. We have seen similar effects of HSP90 inhibitors on other complexes including Chk1/Cdc25C (discussed later; **Fig. 4**) and H-Ras1/Raf1 (data not shown). Thus, PCA provides a useful method to screen small molecule libraries for HSP90 inhibitors, to identify the panoply of cellular proteins that are regulated by HSP90, and to study their regulation in living cells. **Figure 3D** illustrates dynamics of complexes containing Akt1 and PDK1. These complexes reside at the cell membrane in cells grown in serum, and the localization of fluorescence at the plasma membrane can easily be seen in the vehicle-treated cells. Inhibition of PI3 kinase, by drugs such as LY294002, rapidly causes the redistribution of the complex to the cytosol (**Fig. 3D**). This drug effect can be observed within seconds following treatment with LY294002 and is maximal within 7 min. This result also shows that drug effects, which occur “upstream” from a particular interaction can be detected by monitoring interactions “downstream” from the site of action of the drug, an important principle which allows mapping of drugs into pathways by using various interactions as “sentinels” of pathways. **Figure 3E** shows the complexes between the nuclear hormone receptor, PPAR γ , and its coactivator, RXR α . Antidiabetic drugs such as rosiglitazone are PPAR γ ligands that induce the formation of transcriptional complexes containing PPAR γ and coactivators including RXR α (retinoid X-receptor- α). Formation of these complexes precedes transcriptional regulation of genes involved in glucose and lipid homeostasis. These PCAs are useful in screening small molecule libraries to identify other small molecule agonists that regulate various members of the PPAR family. In addition, the selectivity of various lead compounds can be determined by studying the effects of agonists on assays that report the activity of PPAR in association with other coactivators such as SRC-1 and SRC-3, because the preference of PPAR for a particular coactivator is determined by the conformation adopted on agonist binding. Also, similar assays can be constructed for known and orphan nuclear hormone receptors, making this drug target class eminently tractable for novel drug discovery. **Figure 3F** shows complexes of the small GTPase, H-Ras, with the kinase, Raf1. Small GTPases of the Ras and Rho families are among the most studied signaling proteins, and represent promising therapeutic targets for human neoplastic disease. Despite the high level of interest in these proteins, direct analysis of most aspects of Ras protein biology in living cells has not been possible. Much of our knowledge, therefore, has been derived from *in vitro* analyses, or from functional assays reporting on a downstream effect of Ras activity (such as cellular transformation or gene expression). Drug discovery efforts to date have focused on upstream enzymatic regulators of Ras pathway activation (e.g., screens for receptor tyrosine kinase inhibitors) or on Ras posttranslational modification (e.g., farnesyl transferase inhibitor screens) or on downstream kinase-regulated signaling events (e.g., screens for Raf kinase inhibitors). Identification of probes directly regulating Ras family protein activity would enhance our understanding of this area of biology, and possibly lead to identification of novel therapeutic agents. It is notable that the Ras/Raf complexes are

localized at the plasma membrane; Raf proteins are known to associate with the effector domain of active Ras proteins at the plasma membrane (23). By probing the Ras/Raf complex (as compared with, e.g., fluorescently labeled Raf protein alone) this assay focuses exclusively on active signaling complexes. Cotransfection of siRNA targeting H-Ras clearly obliterates the complex. Thus PCA can be used to detect gene silencing in living cells. We recently have applied this approach to detect targets linked to validated drug targets and pathways, where the validated target is used to construct the PCA and the effects of inhibiting individual proteins on the PCA are assessed with high content assays.

5. Quantitative Analysis of Drug Effects on Protein Complexes

When combined with the appropriate image analysis algorithms, PCAs can be used to screen small molecule libraries and to establish dose–response and structure–function relationships. **Figure 4** shows an example of a PCA that reports on DNA damage response pathways. Chk1/Cdc25C complexes are induced by cell treatment with the topoisomerase inhibitor, CPT, for 18 h (**Fig. 4A**). Test compounds that block the pathway from topoisomerase to this signaling node can be identified by preincubating cells with the test compounds preceding treatment with CPT. Two such agents, with different mechanisms of action, are the natural product geldanamycin, and the small molecule kinase inhibitor, Y27632. Chk1 is a client protein for HSP90 (24), and geldanamycin suppresses Chk1/Cdc25C complexes as a result of destabilization of Chk1. These results are easily visualized and quantified, as shown in the plate view and the corresponding dose–response curve (**Fig. 4C**). Y27632, previously described as an inhibitor of Rho kinases, also suppresses these complexes through an unknown mechanism and with a different dose dependency (**Fig. 4C**). Thus, total fluorescence can be used to assess the effects of test compounds acting through these mechanisms. In other cases, however, drugs and toxicants cause changes in fluorescence signal pattern that are not obvious based on total fluorescence. For example, cadmium chloride induces the formation of bright punctuate signals at discrete foci within the nucleus of the cell (**Fig. 4D**). This effect would be masked by measurements of bulk fluorescence but can readily be quantified using algorithms designed to quantify granularity of signal. These examples illustrate the fact that high content methods are capable of detecting not only total changes in signal intensity but also more subtle changes in signal pattern that can occur.

6. Pharmacological Profiling in Living Cells

Even a selective chemical compound that binds to a therapeutic target might have completely unexpected or “off-target” effects when it contacts a living cell, resulting in expensive preclinical and clinical failures. An improved understanding of drug action in living cells would accelerate attrition of compounds (a “fail-fast” strategy) and improve productivity in pharmaceutical research. This goal is best accomplished by profiling lead compounds broadly across cellular targets and pathways in living cells. We, therefore, constructed diverse panels of high content PCAs covering major drug target classes and mechanisms of action and used these assay panels to analyze known drugs and known toxicants, and to perform lead optimization in an iterative fashion. In addition, we have used these assay panels to identify new therapeutic indications for known drugs. The process of evaluating numerous known, marketed drugs, clean and potent lead compounds, and compounds that have failed in preclinical and clinical development has given us a view concerning what constitutes a selective drug vs a nonselective drug. **Figure 5** shows an example of a set of 20 compounds that includes five highly nonselective compounds (1–5) vs other, more selective, lead compounds (6–20). All of these compounds were potent in cells, but the nonselective compounds subsequently failed in animal models resulting from toxicity in various organs. Our data suggests that cellular off-target activity is a common, but underappreciated component of drug failure. In addition to identifying the extent of off-target activity, the underlying pathways contributing to the off-target activity can be pinpointed with these assay panels.

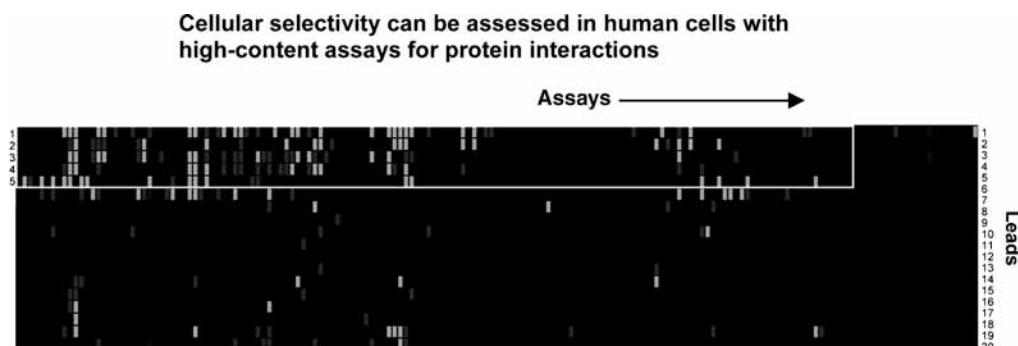


Fig. 5. Cellular selectivity of drugs in human cells. Each of 20 different small molecule lead compounds were analyzed with 191 unique assays in HEK293 cells in 384-well plates, using automated confocal imaging systems (Evotec Opera, Hamburg, Germany). Each assay was a particular protein interaction at a particular time and pretreatment condition. The results are represented as a matrix enabling visualization of significant hits, in which the assays are on the *x*-axis and the compounds are on the *y*-axis. Leads 1–5 were highly nonselective, as is apparent from the number of hits across the assay panel, and these compounds proved to be toxic in preclinical studies.

For example we have seen unintended, off-pathway effects of supposedly selective kinase inhibitors on GPCR pathways. In addition, we have identified new therapeutic uses of known drugs by pinpointing off-target activity on pathways linked to therapeutic efficacy, and by combining PCA with siRNA, we have validated novel drug targets. In sum, these high content assays provide a discovery platform with the potential to impact many areas of pharmaceutical discovery—from target validation to lead optimization—in addition to basic biomedical research.

7. Conclusions

Classical drug discovery paradigms treat protein targets as isolated entities, and pathways as serial and parallel “circuit diagrams.” These concepts do not reflect reality and perpetuate faulty decisions in drug discovery and development. A better understanding of the physical nature of cell signaling and cellular networks, and of drug effects in the context of those networks, is essential to improving drug selectivity and specificity. This growing recognition has led to the call for a pathway-based approach to drug discovery (25).

High-content, cell-based methods enable drug discovery in the context of the biochemical pathways of living cells. We have employed protein complexes as dynamic sensors of cellular pathways. The ability to localize and quantify these events directly in cells allows for the translation of a more realistic view of cell biology into improved productivity of biomedical research and development. As we have shown, these approaches can be adapted to high throughput discovery on a large scale. Moreover, by profiling drugs and lead compounds against a wide array of cellular pathways, we can identify the on-target and off-target activities of drugs in human cells. Strategies such as those described here, and in other chapters in this volume, open the door to a more effective paradigm for drug discovery.

References

1. Stoughton, R. B. and Friend, S. H. (2005) How molecular profiling could revolutionize drug discovery. *Nat. Rev. Drug Discov.* **4**, 345–350.
2. Tian, Q., Stepaniants, S. B., Mao, M., et al. (2004) Integrated genomic and proteomic analyses of gene expression in Mammalian cells. *Mol. Cell Proteomics* **3**, 960–969.
3. Miklos, G. L. and Maleszka, R. (2004) Microarray reality checks in the context of a complex disease. *Nat. Biotechnol.* **22**, 615–621.

4. Remy, I. and Michnick, S. W. (2001) Visualization of biochemical networks in living cells. *Proc. Natl. Acad. Sci. USA* **98**, 7678–7683.
5. Yu, H., West, M., Keon, B. H., et al. (2003) Measuring drug action in the cellular context using protein-fragment complementation assays. *Assay Drug Dev. Technol.* **1**, 811–822.
6. Michnick, S. W., Remy, I., Valois, F. X., Vallee-Belisle, A., Galarneau, A., and Pelletier, J. N. (2000) Detection of protein–protein interactions by protein fragment complementation strategies, Parts A and B, in *Methods in Enzymology*, (Abelson, J. N., Emr, S. D., and Thorner, J., eds.), Academic, San Diego, CA, pp. 208–230.
7. Pelletier, J. N., Remy, I., and Michnick, S. W. (1998) Protein-fragment complementation assays: a general strategy for the in vivo detection of protein–protein interactions. *J. Biomol. Tech.* **10**, 32–39.
8. Galarneau, A., Primeau, M., Trudeau, L. -E., and Michnick, S. W. (2002) A protein fragment complementation assay based on TEM1 β -lactamase for the detection of protein-protein interactions. *Nat. Biotech.* **20**, 619–622.
9. Remy, I., Wilson, I. A., and Michnick, S. W. (1999) Erythropoietin receptor activation by a ligand-induced conformation change. *Science* **283**, 990–993.
10. Remy, I. and Michnick, S. W. (1999) Clonal selection and in vivo quantitation of protein interactions with protein fragment complementation assays. *Proc. Natl. Acad. Sci. USA* **96**, 5394–5399.
11. Remy, I. and Michnick, S. W. (2004) Regulation of apoptosis by the Ft1 protein, a new modulator of protein kinase B/Akt. *Mol. Cell Biol.* **24**, 1493–1504.
12. Remy, I., Montmarquette, A., and Michnick, S. W. (2004) PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. *Nat. Cell Biol.* **6**, 358–365.
13. Remy, I. and Michnick, S. W. (2004) Mapping biochemical networks with protein-fragment complementation assays. *Methods Mol. Biol.* **261**, 411–426.
14. Remy, I. and Michnick, S. W. (2004) A cDNA library functional screening strategy based on fluorescent protein complementation assays to identify novel components of signaling pathways. *Methods* **32**, 381–388.
15. Remy, I., Pelletier, J. N., Galarneau, A., and Michnick, S. W. (2002) Protein interactions and library screening with protein fragment complementation strategies, in *Protein–Protein Interactions: A Molecular Cloning Manual*, (Golemis, E. A., ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 449–475.
16. Spotts, J. M., Dolmetsch, R. E., and Greenberg, M. E. (2002) Time-lapse imaging of a dynamic phosphorylation-dependent protein–protein interaction in mammalian cells. *Proc. Natl. Acad. Sci. USA* **99**, 15,142–15,147.
17. Fang, D. and Kerppola, T. K. (2004) Ubiquitin-mediated fluorescence complementation reveals that Jun ubiquitinated by Itch/AIP4 is localized to lysosomes. *Proc. Natl. Acad. Sci. USA* **101**, 14,782–14,787.
18. Hu, C. D., Chinenov, Y., and Kerppola, T. K. (2002) Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol. Cell* **9**, 789–798.
19. Paulmurugan, R. and Gambhir, S. S. (2003) Monitoring protein–protein interactions using split synthetic renilla luciferase protein-fragment-assisted complementation. *Anal. Chem.* **75**(7), 1584–1589.
20. Luker, K. E., Smith, M. C., Luker, G. D., Gammon, S. T., Piwnica-Worms, H., and Piwnica-Worms, D. (2004) Kinetics of regulated protein–protein interactions revealed with firefly luciferase complementation imaging in cells and living animals. *Proc. Natl. Acad. Sci. USA* **101**(33), 12,288–12,293.
21. Luttrell, L. M. and Lefkowitz, R. J. (2002) The role of β -arrestins in the termination and transduction of G-protein-coupled receptor signals. *J. Cell Sci.* **115**(3), 455–465.
22. Barak, L. S., Ferguson, S. S., Zhang, J., and Caron, M. G. (2001) A beta-arrestin/green fluorescent protein biosensor for detecting G-protein-coupled receptor activation. *J. Biol. Chem.* **272**, 27,497–27,500.
23. Stokoe, D., Macdonald, S. G., Cadwallader, K., Symons, M., and Hancock, J. F. (1994) Activation of Raf as a result of recruitment to the plasma membrane. *Science* **264**, 1463–1467.
24. Arlander, S. J., Eapen, A. K., Vroman, B. T., McDonald, R. J., Toft, D. O., and Karnitz, L. M. (2003) HSP90 inhibition depletes Chk1 and sensitizes tumor cells to replication stress. *J. Biol. Chem.* **278**(52), 52,572–52,577.
25. Fishman, M. A. and Porter, J. A. (2005) A new grammar for drug discovery. *Nature* **437**, 491–493.