Dissecting cellular mechanisms using automated light microscopy.

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As the part lists of living species are being completed from genomic and proteomic research, the behavior of cells can be better described by the integrated activities of their components. Many important cellular processes are mapped to the underlying molecular mechanisms, typically displayed as pathway graphs depicting schematically the flow of events in space and time between cellular components. For example, cell growth signaling pathways start by the activation of receptors at the cell membrane and followed by a cascade of downstream events leading to transcription at the nucleus. However, long-term cellular behavior is a result of flow of signals in networks with redundancy, feedback loops and decision nodes. Cellular decisions are the computational results of these networks, and our ability to model these computations depends on mostly unknown and hard to measure parameters characterizing the functioning of multiple proteins and their interactions.

An alternative method to relate cellular behavior with its components is by perturbing live cells and recording their responses. This approach is known as "reverse engineering" when applied to electronic "black boxes" [1]. The concept is schematically outlined in Figure 1. A wide repertoire of drugs has been long used to perturb and study cellular processes. Drugs can be applied and followed in short times but typically modulate activities of several targets. Genetic manipulations (e.g. by siRNA) are directed to a specific protein, but since the time scale of their action is long, cells often adapt to a specific perturbation via distributed effects. In order to evaluate cell responses, cell shape and morphology are combined with images of cells expressing fluorescent proteins allowing measurement of changes in post-translational modifications, concentrations and sub-cellular localization of each specifically tagged component. This is the key to link cellular phenomena with the underlying molecular mechanism, and a powerful experimental method to dissect normal cell functions and characterize malfunctions in diseases.

Light microscopy has served cell biology as a central experimental technique. Quantitative microscope imaging provides the measurable responses to induced perturbations. The large number of perturbations and cell samples required for screening all possible components mediating complex cellular phenomena calls for adaptation of the microscope into high-throughput automated operation. An important feature we wish to maintain in such screening microscope is the high resolution, which has been the basis for the rich, multi-dimensional information about cells learned using manual microscopy. The cell-based screening system built in our laboratory combines high-throughput with high-resolution imaging. We prepare "reporter cells" expressing fluorescent protein chimeras that label sub-cellular structures of interest, culture them in multi-well plates, subject each well to a different perturbation and screen these plates microscopically at high resolution. The resulted images are processed by quantitative analysis, depicting the cellular changes induced by the perturbations. The system is being employed in collaboration with

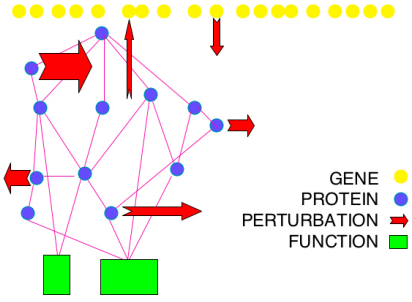

Fig. 1 *A schematic presentation of the multiple perturbation approach to "reverse engineer" cellular networks. Perturbations (red arrows) by genetic manipulations or drugs affect gene expression, protein concentrations, post-translational modifications, sub-cellular distributions etc. modulating protein interactions (the graph arcs) and activities inducing changes in cellular functions (green boxes). The relationship, in magnitude and time between induced changes and functional responses identifies relevant components mediating cellular functions, and help reconstruct quantitative network models for multi-molecular mechanisms.*

Fig. 2 *A photograph of our laboratory, showing the liquid handling robot for culturing cells in multi-well plates, and one of the automated screening microscopes.*

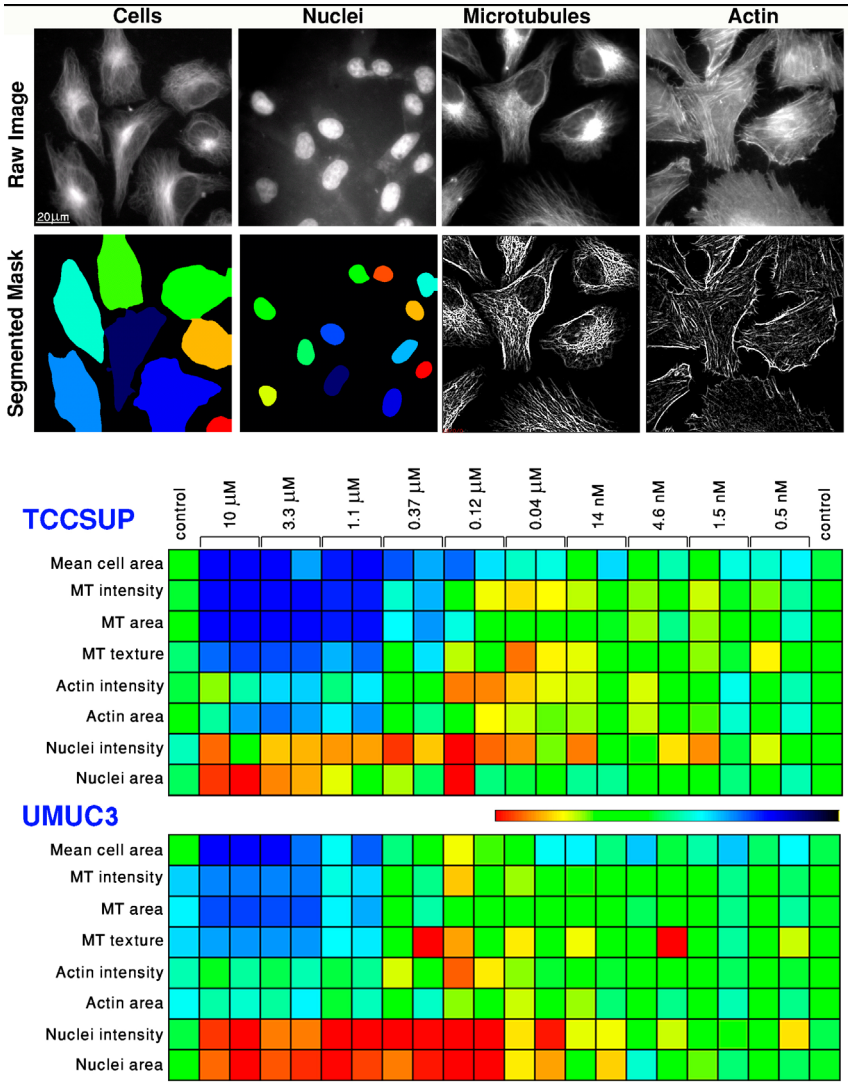

Fig. 3 *Top: The segmentation of individual cells and intracellular features in images, here nuclei actin filaments and microtubules (MT).They are used for quantifying morphological and fluorescent intensity parameters for each cell. 25-100 images are acquired per well. Bottom: Analyzed scores for various parameters calculated from images of two bladder cancer cell lines treated by serial dilutions of nocodazole, a microtubule disrupting drug. The effects are seen by both direct measurements on microtubules and indirect parameters (e.g. cell area, responding to microtubule disruption by cell shrinkage), however, they reflect quantitatively different scores and show systematic cell-line dependence for the same parameter. Increased nuclei intensity and size reflect cell division arrest. Indirect effects on actin are stronger for TCCSUP than for UMUC3.*

the laboratories of Professors Benjamin Geiger and Alexander Bershadsky in screens for the effects of libraries of drugs and siRNA on cell substrate adhesion, cytoskeleton organization, cell migration and cell death. The large number of proteins that assemble in adhesion sites and associate with the actin and microtubule fibers are readily imaged by fluorescence microscopy, both in live cells expressing these proteins tagged by the fluorescent protein color variants and in fixed immuno-labeled cells. The responses of cells to perturbation can therefore be monitored at high spatial, temporal and compositional resolutions [2], reporting with high sensitively about the reorganization of the imaged structures, and indirectly also about the signaling pathways controlling and reading these changes. The basic understanding of these cellular processes is linked with proliferation and apoptosis, locomotion and substrate-dependent anchorage and is important medically in osteoporosis, cancer progression, metastasis, implant compatibility and tissue engineering.

We shall shortly describe our screening microscope systems, (five versions are operating at different departments at the institute), the image analysis pipeline and an example of a recent application with interesting biomedical potential.

1. The screening microscope.

The experimental procedures start by preparation of reporter cells. Such cells stably express fluorescent protein chimeras, and have been tested and selected for uniform morphology and preservation of wild-type properties of importance for the studied applications. For example, for cell adhesion studies the reporter cells stably express one of the abundant components of cell-substrate adhesion structures, (e.g. paxillin) and were selected to be well spread, with uniformly distributed adhesion sites. These cell lines are seeded into 385-well plates by a liquid handling robot [Figure 2].

Each well in this sample plate is treated by a different siRNA or drug at a set of concentrations and times, and the plates are brought to the microscope and imaged by fully automated screening microscopy [3-5]. Imaging at 60x and 100x magnifications use high numerical aperture objectives and provide detailed view of sub-cellular structures "painted" by the live fluorescent tags or after fixation by specific labeling. In order to accumulate good statistics many images are acquired in each well. Up to 5 simultaneous fluorescent colors plus a transmitted light image can be taken. The imaging can be carried for live cells using environmental temperature and CO2 chambers. In special plates we have followed live cells for several days.

The automation generates presently about 1 image/sec, equivalent to 0.3

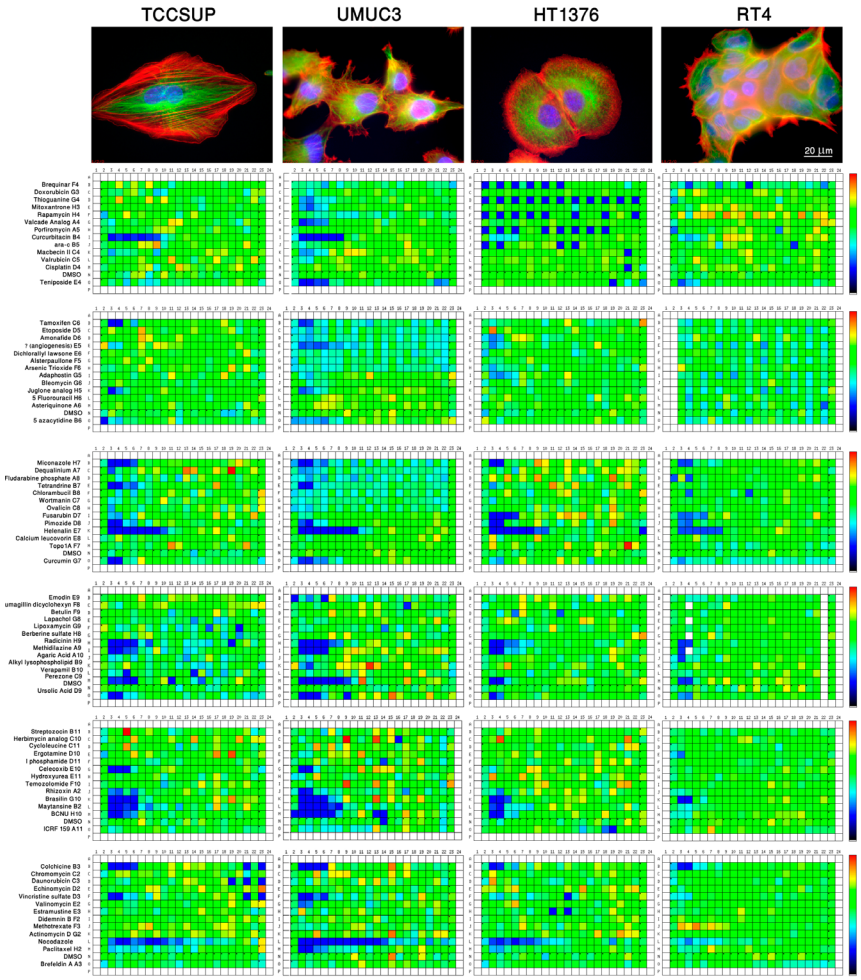

Fig. 4 *Example of results for a high-resolution cell-based screen. The scores displayed here are based on total area covered by cells (red-high blue-low compared to control-green) following incubation for 18 hours with drugs. Each row depicts the effects of serial dilutions of one drug from the National Cancer Institute COMBO library. Four bladder cancer cell lines are shown in the four columns. Typical images of cells fluorescently labeled for actin (red) tubulin (green) and DNA (blue) are shown at the top of the corresponding cell line column. 0.1 TeraBytes of images were analyzed for this figure.*

Terra bytes of image data per week at continuous operation. 50 Terra bytes of NETAPP storage allow fast access for real time visualization of the acquired images (see analysis below).

2. The analysis pipeline.

Over the last decades we have assembled and applied a rich library of quantitative image analysis routines [e.g. 6-8]. However, they were mostly used in interactive image analysis studies. In order to apply such analysis for automated processing of the huge volume of images created in screens we built a new framework that cycles on images to extract the desired biological information. This information is typically presented as morphological and fluorescence intensity parametric descriptors of segmented objects in images, (see Figure 3, top) ,and accumulate this information for the whole experiment, consisting of many plates. Further analysis offers statistical comparisons of the information accumulated in each well with that of the controls. Such comparisons are presented as scores, quantifying the differences from the controls for selected features. As a simple example, the number of cells can be counted in each well. More challenging is to quantify the amount of assembled cytoskeletal fibers as a function of treatments by drugs. Figure 3 (bottom) depicts several such scores for two cell lines treated by the tubulin polymerization inhibitor, nocodazole. Scores are coded by spectral colors: blue implies reduced and red increased effects compared to the green controls.

We have built the analysis pipeline with high degree of modularity for the user, by breaking the process into defined generic steps. Such modularity is usually penalized by inefficient performance and long computation times. We have overcome this problem by allocating memory for fast sharing of data between the analysis modules, and by implementation of fast processing algorithms, some of them developed by the members of the Weizmann applied mathematics department. Processing is sufficiently fast to be monitored and optimally tuned for each application in real time using standard workstations, and then run on our computer cluster, processing all the data acquired in a screen.

3. Diversity of bladder cancer cell responses to drugs.

Cancer diversity imposes a major problem for reliable diagnostics, prognosis and optimization of therapy. Presently cancers are graded by histopathology, by the increasing number of mutated genes identified in cancer cells, and by the abnormal expression levels of cancer related proteins. Cell-based screening microscopy is potentially capable of testing experimentally cell responses to chemotherapy and other cancer treatments. We have subjected four different cell lines derived from bladder cancers to the NCI COMBO library of 77 drugs used medically and in different phases of clinical trials. Figure 4 shows the analyzed scores summarizing the doze-dependent effects of these drugs on the four cell lines. This data displays differential cell-line dependence that changes with the drug. Collaboration with clinicians aims at the development of methodologies that will be able to supply predictive inputs in personalized optimization of drug cocktail applied by chemotherapy.

4. Development of new microscope methodologies.

Tissue culture cells do not always provide faithful models for multi-cellular mechanisms in organs. Therefore, embryos and small organisms (e.g. fish, flies, warms) attract increasing interest as models for human diseases. They would provide important inputs from screens for drug effects at the super-cellular and physiological levels. Light microscopy at high resolutions is confronted by inherent limitation when applied to thick specimens such as tissue biopsies or small organisms. Adaptive optics is a technology developed by the astronomers for correcting the aberrations induced by atmospheric turbulence in large terrestrial telescopes. It was recently applied for correcting optical aberrations in microscopy due to specimen thickness. We have also shown that special adaptive elements allow fast focusing without specimen movement [9]. This technology will help fast three-dimensional screening of biopsies and other thick specimens such as C. eleganse.

Selected publications

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