Innovations

Shine a light
Aurora Biosciences Corporation

Drug companies are faced with an embarrassment of riches these days: huge libraries of compounds made by combinatorial chemistry, and a multitude of new drug targets emerging from genome sequencing. The science of screening — picking out the one compound that matches the one target — is now the limiting factor.

The solution appears to be more miniaturization and more automation. Both of these concepts are part of the ultra-high-throughput screening system (UHTSS) under development at Aurora Biosciences Corporation (La Jolla, CA, USA). Aurora is young and small, and it is competing with all the large pharmaceutical companies, which have their own approaches to high-throughput screening. But a combination of sensitive fluorescent detectors, general-purpose screens using living cells, and a complex web of collaborations could give the UHTSS the edge it needs to succeed.

Divorcing fluorescent couples

Roger Tsien, of the Howard Hughes Medical Institute and University of California, San Diego, USA, is one of the founders of both Aurora and the discipline of fluorescent detector engineering. Beginning in 1975, Tsien designed a series of synthetic calcium detectors including BAPTA, fura-2, indo-1 and fluo-3.

Aurora bases its most important assay for the UHTSS around a new gene expression indicator named CCF2/AM. Although the detailed structure of this compound has not been disclosed, Aurora's Chief Technical Officer Gordon Foulkes says that it consists of two fluorophores connected with a cephalosporin linker, and esterified so that it crosses membranes and is trapped inside live cells as the polyanion CCF2. The first fluorophore of CCF2 absorbs low wavelength (violet) light and would, by itself, emit blue light. But if CCF2 is intact, the excited state energy is transferred to the second fluorophore and re-emitted as green light. This energy relay relies on a quantum mechanical effect called fluorescence resonance energy transfer (FRET), which only works over distances of a few nanometers.

The UHTSS uses live cells with a β-lactamase reporter gene. If the reporter is active, the β-lactamase cleaves the cephalosporin linker and the fluorophores are separated. FRET no longer occurs, so the fluorophores emit more blue light and less green light.

The two parts to the response — more blue light and less green light — allow the result to be expressed as a ratio. This cancels out variability caused by unequal loading of CCF2/AM or differing numbers of cells, and means that duplicate or triplicate assays are unnecessary.

By simply changing the promoter sequence driving the production of β-lactamase, the assay can be adapted to test for the effectiveness (or, using stress-induced promoters, the toxicity) of any number of therapeutic agents. "We've tried to create a one-stop shop for screening," says Foulkes. Each new cell line, responsive to activation of one of the major signal-transduction pathways in the cell, is selected using a fluorescence-activated cell sorter.

Assay adaptability is one thing that is missing in enzymatic assays, which must be customized for each new target. Protein-binding assays are simpler, but do not differentiate between true inhibitors, true activators, and inert binders.

The Aurora assays, says Tsien, do that and more: they can target an entire pathway, including components that are yet to be defined. "You may not know which macromolecule is best to attack," he explains. "If you are doing an enzyme assay, you have to make that choice and put all your eggs in that one basket."

More potential targets does not mean more hit compounds. "Cell-based assays provide a potentially very valuable sieve," says Tony Czarnik of Irori Quantum Microchemistry. "If you really have access to millions of compounds, conventional assays will give you more actives than you can deal with." A cell-based assay can filter out many of these compounds, including those that kill cells, do not get into cells, or do not work inside cells. Unfortunately, says Czarnik, "the sieve is a very inexact one," and results in one cell line may not match up with results in another.

The pathway downstream of G-protein-coupled receptors (GPCRs) is one of the many pathways that can be tested using the β-lactamase system. Aurora is making such assays generic by using the "promiscuous" G proteins ($G_{\alpha 15}$ in mice and $G_{\alpha 16}$ in humans) discovered by Mel Simon at Caltech. These G proteins are activated by a wide variety of GPCRs, and so can be used to bypass the selectivity of the receptor–G-protein interaction.

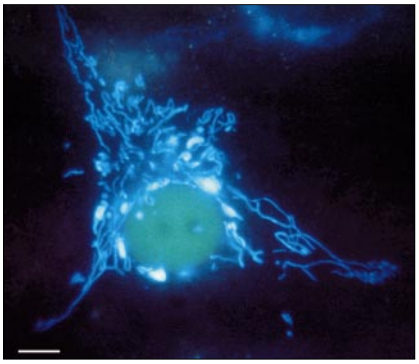
A protein of many colors

With the cloning of the gene for green fluorescent protein (GFP) in 1992, and the demonstration that it works in many different organisms in 1994, fluorescence detection entered a new era. Now the detector was made by the cell not the chemist, and it could be improved by molecular biology.

GFP, a product of the Pacific Northwest jellyfish *Aequorea victoria*, is remarkable for at least two reasons.

First, says Tsien, "it has learned to do surgery on its own guts to make the chromophore," the unit of fluorescence. After the protein is synthesized, a group of three amino acids spontaneously cyclizes and then is oxidized to yield a *p*-hydroxy-benzylidene imidazolinone group with a delocalized pi electron cloud.

Figure 1



Variants of GFP can be used to detect protein localization, in this case to the nucleus and mitochondria. Image courtesy of Rosario Rizzuto, from *Curr. Biol.* (1996) 6, 183–188.

And second, "the rest of the protein forms a rigid shell around the chromophore," says Tsien. This means that the light energy that the chromophore absorbs is not dissipated, but rather is efficiently re-emitted as the fluorescent signal.

But GFP, in its original form, has its problems. It takes too long to make the chromophore, it is not very bright, and it absorbs at two different wavelengths, with the lower wavelength form isomerizing to the higher wavelength form over time.

Tsien's lab, including Andrew Cubitt and Roger Heim who are now both at Aurora, solved all three problems to some extent with a serine to threonine conversion in the chromophore. The new protein makes the chromophore more rapidly, and only absorbs at the higher wavelength, at which it is much brighter. Other changes have resulted in blue, cyan and yellow fluorescent proteins. A number of these changes have been patented and licensed to Aurora.

Tsien felt that GFP, although ~1000 times less sensitive as a cellular reporter than the β-lactamase system, still had potential. "The aim is not just to make it better, it's to teach it a lot of new tricks," he says.

The first trick was to make a protease sensor by joining the blue and green proteins with a 25 amino acid linker. GFP itself is resistant to proteases, but cleavage of the linker disrupts FRET between the two proteins. Tsien and Aurora also created a GFP kinase sensor, which changes its fluorescence upon phosphorylation, and with others have used GFP to detect protein localization (Figure 1) and interactions between proteins (using two GFP fusions and FRET).

FRETting over ions

The β-lactamase and GFP systems are not easily adaptable to measuring the opening and closing of ion channels, and electrodes are impractical for high-throughput screening. Tsien's solution to this problem, working with Tito Gonzalez (also now at Aurora), is again based on FRET. In this case the donor is coumarin linked to the membrane lipid phosphatidyl-ethanolamine, and the acceptor is a synthetic oxonol in the membrane. At normal resting potentials FRET is efficient, but when the cell is depolarized the negatively charged oxonol migrates across the bilayer while the labeled lipid remains in the outer leaflet, thus diminishing FRET.

Nanoplates and robotics

About half of the technical staff at Aurora are engineers, a good indicator that the machinery is as important as the assay methods. In two or three years, Aurora aims to have its UHTSS fully operational, screening 100,000 compounds a day using 3456-well "nanoplates" that are the same size as conventional 96-well plates. Each well of a nanoplate only holds ~1 μl (including tens to hundreds of cells). A piezoelectric device delivers volumes as small as 200 pl by using high frequency voltage pulses to create 2000 drops per second. The tiny volumes will obviate the need for serial dilutions of compounds before they are added to assays. Packard Instrument Company is assisting with the development of the nanoplates, the fluid delivery system, and a high density, sensitive fluorescence detector.

Aurora is trying to protect every component of its system with a series of patent applications, which are jostling with applications covering other systems from other companies. According to Czarnik, with no screening system established as the dominant one, "we're in the wild west right now."

While the UHTSS is being developed, Aurora is using prototype versions for the screening of ~10,000 single compounds a day, using standard 96-well plates. Already the throughput is high enough to dispense with the complications of compound mixtures. "Mixtures seemed like a good idea early on [in the history of combinatorial chemistry]," says Foulkes, "but by the time you can screen as fast as we can, single compounds make a lot more sense."

The collaboration game

Packard is far from Aurora's only collaborator. Foulkes is seeking four or five partners to share the cost and benefits of the UHTSS, and so far Bristol-Myers Squibb and Eli Lilly and Co. have signed up. "No one company could develop an equivalent system for the cost that it takes to be part of the consortium," he says.

In the meantime, other help is coming from all over: discrete-compound combinatorial libraries from Alanex Corp. and Arqule, Inc.; drug targets from genomics companies Sequana Therapeutics, Inc. and Allelix Biopharmaceutical Inc.; robotics from Carl Creative; and storage and retrieval systems from Universal Technologies, Inc.

This multipartner marriage will need a lot of care and attention before profits start flowing. But if UHTSS becomes the screening method of choice, the payoff will be enormous.

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