Making connections

Technological innovations in detecting and studying protein–protein interactions are providing new ways of doing research in cell signalling. Diane Gershon investigates.

Cell-signalling research aims to understand how cells convert extracellular signals into the required cellular responses, and how these pathways can go awry and cause disease. With the emergence of cell signalling as a significant field in its own right over the past few years, a dizzying array of reagents, assays and technologies are now available to help researchers (see 'In the market place').

Cell-surface receptors

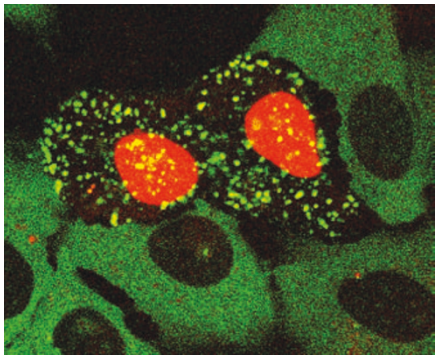
The large and diverse family of G-protein-coupled receptors (GPCRs) responds to a wide range of agonists, including amines, hormones, neurotransmitters and even light. This sheer diversity makes GPCRs a rich source of potential drug targets.

"We spent a lot of the 1990s, as did others, isolating novel genes encoding these GPCRs, which essentially had eluded classical pharmacology," says Brian O'Dowd of the department of pharmacology at the University of Toronto, Canada. But many of these are still 'orphan' GPCRs, for which a natural ligand has not been identified.

To try to tap their therapeutic potential, O'Dowd's group has developed a multipurpose cellular assay (MOCA) that can be used to screen for compounds that activate or block any GPCR, and that needs no prior knowledge about the G proteins or second messengers involved. O'Dowd's lab jokingly refers to this as the 'mother of all cellular assays'. The technology is being commercialized by Toronto-based PatoBios, of which O'Dowd is a co-founder.

The GPCR is genetically modified to incorporate a nuclear-localization sequence, which causes the receptor to be internalized and translocated to the nucleus, from which it is unable to recycle to the cell surface. The interaction of a ligand with the modified GPCR prevents translocation, and the receptor is retained on the cell surface. The different distributions of the receptor can be visualized by various methods such as fluorescent tags. The assay can also be adapted to detect ligand binding to the important family of transporter proteins involved in the re-uptake of neurotransmitters.

Norak Biosciences of Research Triangle Park, North Carolina, has also developed a cell-based fluorescence assay for finding ligands for orphan GPCRs. Its Transfluor technology detects the binding of β-arrestin to the cytoplasmic part of the receptor, which occurs only after a ligand has bound. Activated receptors can then be detected and isolated. The assay has been validated on various image analysers, including the IN Cell Analyzer 3000 from GE Healthcare, Little Chalfont, UK, the ArrayScan from Cellomics, Pitts-

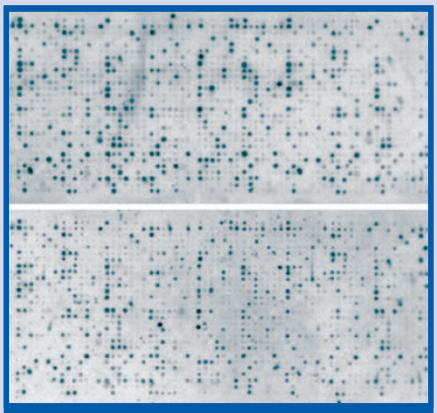


R.H. OAKLEY/NORAK BIOSCIENCES

Activated receptors show up as dots.

IN THE MARKET-PLACE

Finding the right reagent or assay for the job in hand can be a daunting prospect in a field as complex as cell signalling. Some companies are taking a one-stop shopping approach; others are pursuing niche markets, specializing in, for example, antibodies, peptide substrates for kinases and proteases, enzymes, especially protein kinases, or protein and peptide microarrays, such as those from Sigma-Aldrich in St Louis, Missouri, PepScan Systems in Lelystad, the Netherlands, and Zeptosens in Witterswil, Switzerland. Even companies usually thought of as having a molecular biology focus are getting in on the act, as evidenced by the acquisition of Molecular Probes in Eugene, Oregon, a leader in novel fluorescence-based technologies for labelling biological molecules, by Invitrogen of Carlsbad, California in 2003.



PEPSCAN: DATA COURTESY OF J. TUYNMAN & D. RICHEL ACADEMIC MEDICAL CENTER, THE NETHERLANDS

PepChip protein kinase profiles of normal (top) and tumour tissue.

"I think it is fair to say that it's becoming increasingly difficult to be totally comprehensive in signal transduction," says Konrad Howitz, director of molecular biology at BIOMOL in Plymouth Meeting, Pennsylvania. BIOMOL, now in its twenty-first year of operation, was one of the first companies to specialize in this area and to use signal transduction as a theme for a catalogue.

BIOMOL's recent product development has been aimed at developing enzymes, substrates, antibodies and inhibitors for the study of histone deacetylases, which play a role in the regulation of gene transcription and other biological processes involving chromatin, and sirtuins, which are protein deacetylases implicated in the regulation of ageing. BIOMOL recently merged with UK-based Affiniti Research Products, and now offers the Affiniti line of ubiquitin and proteasome research tools.

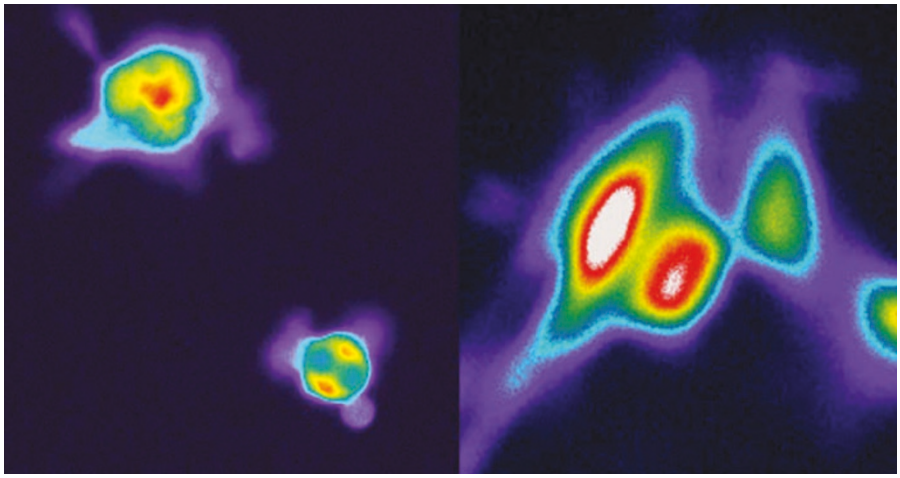
"We still find that the model of niche excellence is the one that appeals to us and our customer base," says Ian Ratcliffe, president and chief operating officer of Upstate in Charlottesville, Virginia, another established company supplying cell-signalling tools. Ratcliffe says Upstate's customers are evenly split between academia and industry.

"We think of ourselves as a 'content' company," says Ratcliffe, and so is always on the lookout for companies with novel detection technologies. One such is CIS bio, based in Bagnols-sur-Cèze, France, which sells reagents and assays based on time-resolved homogenous fluorescence resonance energy transfer (HTRF) for probing molecular interactions. The company's proprietary HTRF technology uses a luminescent europium (Eu^{3+})-based cryptate compound as donor to tag one of the interacting partners and a phycobiliprotein (XL665) as acceptor to tag the other. When the two partners

burgh, Pennsylvania, and the Opera high-throughput confocal microscopy platform from Evotec of Hamburg, Germany.

Blocking on cue

The intracellular interaction between the GPCR and its associated G protein is also a potential drug target. This is the approach of drug-discovery company cue BIOtech of Evanston, Illinois, which is also marketing its Minigene vectors. These deliver small peptides that block signal transduction through a specific G protein by competing for the site where the G protein would normally bind to the receptor. "You can turn off each G protein individually inside the cell," says Annette Gilchrist, president and founder of cue, which licensed the technology from Northwestern University. The company offers both plasmid-based cDNA vectors for transient transfection and retroviral vectors for hard-to-transfect cells.



CUE BIOTECH

Cells treated with a cue BIOtech Gq Minigene vector (left) respond less strongly than normal cells (right) to stimulation by thrombin.

Pure and simple

Many potential drug targets, such as GPCRs and ion channels, are transmembrane proteins, and their isolation and study is difficult as they often need to remain embedded in a lipid membrane to maintain their structural integrity.

By exploiting the fact that cell-membrane proteins are incorporated into the surface envelope of budding retroviruses, Integral Molecular of Philadelphia, Pennsylvania, has developed a way of isolating membrane proteins from cells while preserving their native structure. Cells producing non-infectious retroviral particles are also engineered to make high levels of the desired membrane protein. As the virus core buds from the cell it is surrounded by cell membrane enriched with the protein. The resulting Lipoparticles marketed by the company are non-infectious spheres (100–150 nm in diameter) of retroviral core protein surrounded by a phospholipid bilayer containing around 100 molecules of the desired membrane protein in its native conformation. The particles are stable when frozen and refrigerated. "Generally, the preparations that we make are greater than 100 picomoles of membrane protein per milligram of total protein," says Benjamin Doranz, president, chief scientific officer and founding partner of Integral Molecular.

"The most complex protein that we've obtained is a 14-spanning membrane protein amino-acid transporter," says Doranz. But there will be limits, he says, such as membrane proteins with extremely long cytoplasmic tails that interfere with viral assembly or those that do not traffic to the cell surface.

Lipoparticles can serve as a source of homogeneous and structurally intact membrane proteins for high-throughput screening, monoclonal antibody production and structure analysis using X-ray crystallography. They are also being paired with optical biosensors, such as the surface plasmon resonance (SPR)-based detectors developed by Biacore, of Stockholm, Sweden, for the kinetic analysis of membrane protein interactions with antibodies and ligands.

Characterizing kinases

Protein kinases are garnering increasing attention as drug targets, particularly in the light of the recent success of Gleevec, a tyrosine kinase inhibitor developed by Swiss-

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bind, fluorescence resonance energy transfer (FRET) can be induced between the cryptate and XL665, which emits a long-lived fluorescence at 665 nm. CIS bio entered into partnerships with both Upstate and antibody company Cell Signaling Technology of Beverly, Massachusetts, in August this year — a move that will extend the reach of its HTRF technology in the life sciences. CIS Bio also recently linked up with detection-system manufacturers BMG LABTECH of Offenburg, Germany, Molecular Devices of Sunnyvale, California, and Tecan of Zurich, Switzerland, to develop HTRF-compatible fluorimeters or upgrade kits for optimizing existing equipment. Upstate is also collaborating with Dharmacon of Lafayette, Colorado, to develop small interfering RNA (siRNA)-based products as an alternative to gene knock-outs, and with Evident Technologies on the quantum dot front (see 'Quantum dots begin to show their true colours', page 247).

In September, Serologicals Corporation, which also owns Chemicon of Temecula, California, announced plans to acquire Upstate. Ratcliffe sees it as a good fit, combining the company's strengths in phosphorylation, nuclear function, drug discovery and screening with Chemicon's expertise in developing tools for neurobiology. "There's little overlap but very complementary ranges of products," he says.

ZEPTOSENS

ZeptoSens cell lysate array.

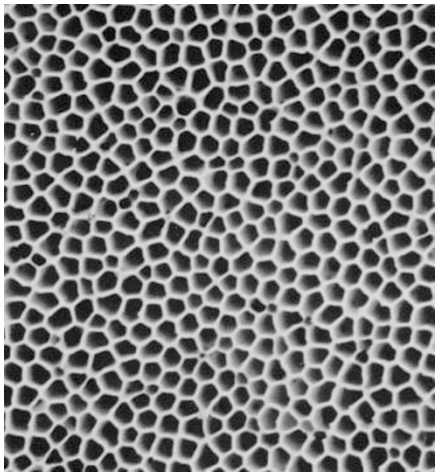
Companies such as Amersham Biosciences, now part of GE Healthcare, aim to offer integrated solutions. "We're looking at this holistically and we're not interested in just providing a small part of the process. We're interested in providing innovative solutions that go across the biology, the hardware and software and into the informatics," says John Anson, vice-president of development at GE Healthcare Biosciences. These products range from image analysers for cell-based assays (IN Cell Analyzer 1000 and 3000 and LEADseeker) to pH-sensitive cyanine dyes for tracking the internalization of cell-surface receptors and GFP-based live-cell translocation assays developed in collaboration with BioImage of Soeborg, Denmark. **D.G.**

based pharmaceutical company Novartis, which was approved for the treatment of chronic myelogenous leukaemia in 2001. Gleevec represents a new class of drugs that disrupt components of the intracellular signalling pathways that cause cells to grow and divide uncontrollably, giving rise to cancers.

There are more than 500 protein kinase genes in the human genome. They catalyse the specific phosphorylation of proteins and play an essential role in many signalling pathways, including those involved in cell-cycle control.

One way of evaluating the substrate selectivity and function of protein kinases in a high-throughput format is by peptide microarrays to which the kinases will bind selectively. Pepscan Systems of Lelystad, the Netherlands, launched its PepChip Kinase peptide microarray about a year ago. "Currently, we have 1,150 different substrates," says Jos Joore, vice-president of array technology at Pepscan, all derived from a public database. The microarray can be used for substrate profiling of known and unknown kinases and for specificity testing of kinase inhibitors. The company is also turning its attention to proteases. Longer term, Joore says, it is trying to improve the specificity of its peptide substrates by providing them as constrained loops, or a combination of constrained loops, rather than linear peptides.

The three-dimensional surface of the PamChip from PamGene of 's-Hertogenbosch, the Netherlands, is designed to allow



PAMGENE

The porous PamChip surface provides a greater area for protein immmobilization.

peptide substrates to be deposited at higher concentrations than conventional arrays. "We can immobilize much more material per square millimetre than other flat materials," says Rob Ruijtenbeek, PamGene's head of kinase research. A 500-fold increase in reactive surface compared with two-dimensional arrays is claimed.

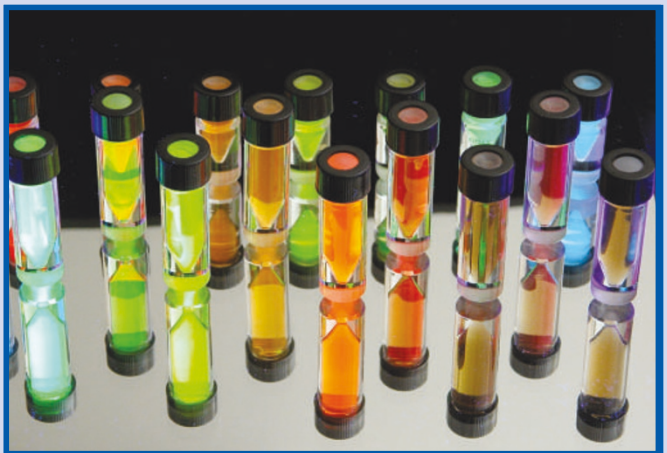
At the heart of the system is PamGene's 5D-Pulse flow-through microarray technology, in which peptides are covalently immobilized using inkjet technology onto the porous microarray surface via the peptide amino terminus. Sample is then pumped back and forth through the porous material to facilitate mixing. The pumping-cycle frequency can be changed and detection is with fluorescent antibodies using a CCD camera/microscope. The system provides kinetic readouts in which substrate conversion is monitored over time; traditional array formats limit detection to a single time point.

In June, PamGene announced plans to join forces with Jerini Peptide Technologies (JPT) in Berlin, Germany, which allows PamGene to marry its microarray platform with JPT's comprehensive peptide sets for kinase profiling. Zeptosens of Witterswil, Switzerland, is similarly increasing the range of its arrays by selling antibodies developed by Cell Signaling Technology of Beverly, Massachusetts, for use with the Zeptosens planar waveguide detection protein microarray platform. With this technology, only fluorophores located at or near the surface of the waveguide are excited and signals from unbound molecules in the bulk solution are not detected.

According to Peter Oroszlan, director of business development at Zeptosens, this technology provides a significant increase in signal-to-background ratios, enabling detection of low-abundance proteins such as signalling molecules. "Our system allows you to measure 600 protein molecules in a spot, which corresponds to one zeptomole [10^{-21} moles]," he says. ZeptoMARK protein microarrays are available in capture or reverse-array formats (see *Nature* **429,** 102; 2004), and applications include expression monitoring of proteins during drug profiling and pathway mapping,

QUANTUM DOTS SHOW THEIR TRUE COLOURS

As an alternative to organic dyes, quantum dots (QDs) may be worth a closer look. These semiconductor nanometre-sized crystals, typically with a cadmium-based core, avoid some of the shortcomings associated with traditional organic dyes and fluorescent proteins (see *Nature* **413,** 450; 2001). QDs are brighter, not prone to photobleaching, and come in a wide range of colours. By changing the size of the particle, the emission can be tuned to any wavelength, from ultraviolet to infrared. QDs also have narrow emission spectra, which means more colours can be used at a time with minimal channel overlap. Multiple colours of QDs can be simultaneously excited using a single light source. QDs have, for example, been used to to track the activity and diffusion of individual glycine receptors in the neuronal membrane for up to 20 minutes — considerably longer than is possible with Cy3 dyes.



Evident Technologies' quantum dots come in many colours.

"We don't expect QDs to completely displace organic dyes," says Clint Ballinger, chief executive of Evident Technologies in Troy, New York. "They make an attractive alternative for many different applications." Evident began by making ultrafast optical switches for the telecom industry. But when the bottom fell out of that business, the company turned to alternative applications for its nanocrystals. It sells its QDs (EviDots) as EviTags, which are water-stabilized, conjugation-ready QDs that can be coupled to proteins or antibodies, and as EviFluors, which have biotin groups.

Another company with know-how in this area is Quantum Dot Corporation (QDC) of Hayward, California. Its product line includes basic Qdot nanocrystals, Qdot bioconjugates coupled to proteins, oligonucleotides and small molecules (streptavidin, protein A and biotin), and Qbead microspheres. In June, QDC introduced a kit that enables researchers to conjugate their own antibodies to Qdot nanocrystals — a procedure that the company says typically takes 3–4 hours. The kit can also be used to couple other thiol-containing molecules to QDs.

So what of the future? "Some customers don't like cadmium," says Ballinger, and Evident plans to launch indium phosphide-based EviDots in the next few months as an alternative to cadmium for the visible range. **D.G.**

monitoring activation-state markers, such as phosphorylated proteins, and the study of disease progression.

Read-out

Biosensors for tagging proteins to monitor their activation levels and distribution continue to get more sensitive and versatile. The impact of green fluorescence protein (GFP) and its variants in studying cell signalling is immense. Biosensors that use GFP can, for example, detect conformational changes in proteins in response to ligand binding, changes in protein localization or changes in protein activity.

New fluorescent dyes are also being developed. Klaus Hahn and his colleagues at the University of North Carolina School of Medicine, Chapel Hill, recently reported a biosensor that can visualize the natural dynamics of an unlabelled endogenous intracellular signalling protein, the GTPase Cdc42, in living cells. The sensor is composed of the Cdc42-binding fragment of the Wiskott–Aldrich syndrome protein (WASP), to which a novel merocyanine dye has been coupled. The dye is sensitive to changes in hydrophobicity that occur at the interface between the interacting proteins. Binding of WASP to GTP-activated Cdc42 causes the dye to fluoresce. The sensitivity provided by direct excitation of a novel fluorescent dye enables detection of protein activation at native levels.

One of the more novel detection platforms is quantum dots (see 'Quantum dots show their true colours', page 247); another is single-molecule photon stamping spectroscopy, which is being used to study the dynamics of the interactions of single proteins (see 'Probing real-time protein interactions').

Lighting-up protein interactions: Klaus Hahn (left) and Alexei Toutchkine with the dye on which their biosensor is based.

LI-COR Biosciences of Lincoln, Nebraska, offers two-colour near-infrared fluorescence detection of signal transduction events. The firm originally applied its infrared detection technology to western blots, "but a western blot is not particularly convenient for looking at a pathway", says Michael Olive, LI-COR's vice-president of research and development. The company has developed the In-Cell Western assay for quantifying proteins in fixed cultured cells in 96- or 384-well microplates in less time than conventional western blots by bypassing the need for lysate preparation and the use of gels and membranes. The use of two spectrally distinct near-infrared dyes effectively doubles the number of endpoints that can be analysed, enabling, for example, the measurement of both phosphorylated extracellular signal-regulated kinases (ERKs) and total ERK protein at the same time.

With slight tweaking, the 'In-Cell Western' can become the 'On-Cell Western'. This was developed by James Wager-Miller in the department of anaesthesiology at the University of Washington in Seattle, who is using it to follow the internalization and recycling of GPCRs, in particular the cannabinoid receptor 1, to and from the cell surface. The hope is that this will lead to a better understanding of how these trafficking events can lead to the desensitization of cells following prolonged or repeated exposure to agonists.

The In-Cell Western is amenable to automation and this month the company will launch a new two-colour plate reader called Aerius, which automates the assay. This may take the technology "into the realm of lead validation", says Olive, helping to prevent costly drug failures later on.

The next challenge for cell signalling will be to look at cellular behaviour on a global scale and for this further improvements in technology will be needed, along with better computational and mathematical tools for deriving information about complete signal-transduction networks. ■

Diane Gershon is assistant editor, *Nature Medicine* Technical Reports.

PROBING REAL-TIME PROTEIN INTERACTIONS

Interactions between proteins are one of the main ways in which signals are transferred onward in intracellular signalling pathways. But few methods of studying these interactions can capture the dynamic nature of molecular recognition within signalling complexes.

A group led by Peter Lu, senior research scientist in the W.R. Wiley Environmental Molecular Sciences Laboratory at Pacific Northwest National Laboratory in Richland, Washington, has now developed a method to do just that.

Using a technique called single-molecule photon stamping spectroscopy, which detects and analyses photons that are emitted as single proteins interact, the group has been able to capture proteins in motion as they flip-flop against each other in a manner somewhat analogous to line flicking in fly-fishing. "This type of behaviour cannot be measured by static structure measurements such as X-ray crystallography or NMR," says Lu.

His team has used this approach to study the interaction of single molecules of activated Cdc42 with the dye-labelled WASP (Wiskott–Aldrich syndrome protein) biosensor developed in collaboration with Klaus Hahn and his colleagues (see main text above). Their results indicate highly dynamic — rather than static — interactions between this pair of signalling proteins.

Lu's set-up is largely 'home-built'. Measurements of the interaction of single molecules of Cdc42 with the dye-tagged WASP fragment are made using confocal fluorescence microscopy with laser excitation. Fluorescence photons are directed towards a photon-stamping detector, which records the intensity and duration of the photon peaks; the data are then analysed to determine the dynamics of the protein–protein interaction.

Peter Lu studies the dynamic interactions of signalling complexes.

Technical limitations at present are photobleaching of the dye molecule by the excitation laser and movement of the complex away from the laser focal point. Lu is experimenting with methods for confining the complex either using agarose gel or by tethering one of the protein partners to a glass surface.

Lu plans to extend the single-molecule spectroscopy approach to other important biomolecular complexes under physiological conditions, ultimately hoping to study single-molecule protein conformational dynamics in living cells. "Right now, the most interesting part may not be the technique but the new scientific information we learn," he says. **D.G.**