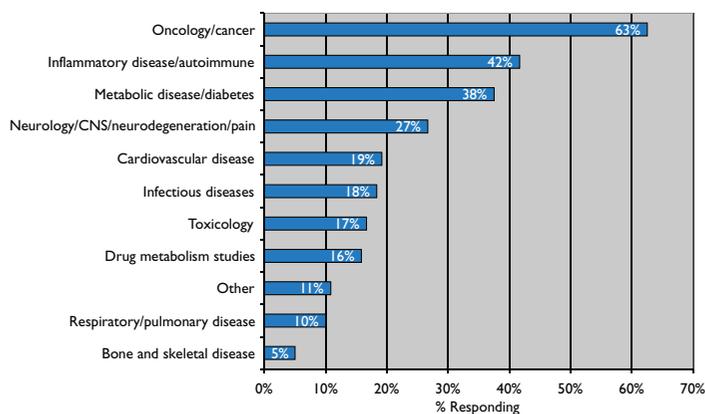**Pathway Analysis**

PATHWAY ANALYSIS
understanding the bridge between environmental cues and cellular response

By Dr John Comley

Understanding the function of a protein in the context of normal and abnormal cellular processes requires a comprehensive knowledge not only of its regulation but also of its role in signalling and metabolic networks in the cell. Malfunctioning signalling pathways can lead to a variety of pathologies, hence why they are particularly investigated in the oncology/cancer disease area. The protein classes of greatest interest in pathway analysis are transcription factors and tyrosine kinases/adaptors, with PI3K/Akt, apoptosis, MAPK and NFkB signalling particularly targeted. Platform technologies most used to investigate pathway analysis today include western blot real-time PCR, reporter gene, siRNA and ELISA. The preferred experimental approach is by pathway, with efficacy ranked as the most important driver for investigating pathway analysis. Informatics tools play a key role in building pathway models to analyse and interpret experimental data from pathway analysis research. In this review, vendor updates highlight some of the latest tools available for signalling pathway analysis. Collectively these tools can be expected to facilitate rapid profiling across many different signalling pathways to help validate lead compounds, determine dose-response relationships, indicate whether signalling feedback or cross-talk is interfering with a compound's effectiveness, and pinpoint potential off-target effects. Software analysis of this experimental data in the context of known biology can then narrow in on what is most important in datasets and discover novel relationships. The identification and characterisation of the components that make up the cellular signalling pathways will unquestionably remain a subject for in-depth investigations in the years to come.

Figure 1: Disease areas investigating/targeting with pathway analysis

Disease area	% Responding
Oncology/cancer	63%
Inflammatory disease/autoimmune	42%
Metabolic disease/diabetes	38%
Neurology/CNS/neurodegeneration/pain	27%
Cardiovascular disease	19%
Infectious diseases	18%
Toxicology	17%
Drug metabolism studies	16%
Other	11%
Respiratory/pulmonary disease	10%
Bone and skeletal disease	5%

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ignal transduction pathways are the bridge that links extracellular environmental cues (eg the binding by a specific cell surface receptor of a hormone, cytokine, growth factor, ligand etc) and the cellular response. These signalling pathways are composed mainly of proteins that can interact, move to specific cellular locations, or be modified or degraded. Protein phosphorylation via the activation of a protein kinase and transfers a phosphate from ATP to an inactive molecule of another protein kinase molecule is a major mechanism of signal transduction. The integration of these events often leads to the activation or inactivation of transcription factors, which then induce or repress the expression of thousands of genes. Alternatively, a component of a kinase pathway may move to the plasma membrane to act on a substrate substituent of an ion channel. Because of this critical role in translating environmental cues to cellular responses, malfunctioning signalling pathways can lead to a variety of pathologies. It is hoped that the identification and characterisation of the components that make up the cellular signalling pathways' underlying diseases, such as cancer, will lead to developing new treatments.

HTStec's recent pathway analysis (signal transduction) trends survey and report, published in September 2011, set out to learn how and where pathway analysis assays were being investigated today[1]. In this review we will look at some of the report's finding and contrast them to the latest vendor offerings in pathway analysis tools.

Disease areas investigating

The majority (63%) of survey respondents were investigating/targeting pathway analysis in the oncology/cancer disease area. This was followed by inflammatory disease/autoimmune (42% utilising); metabolic disease/diabetes (38% utilising); neurology/CNS/neurodegeneration/pain (27% utilising); and then cardiovascular disease (19% utilising). Least respondents were investigating/targeting pathway analysis in the respiratory/pulmonary disease or bone and skeletal disease (**Figure 1**).

Protein classes of interest

The protein class of greatest interest to survey respondents with respect to pathway analysis was transcription factors (63% investigating). This was closely followed by tyrosine kinases/adaptors (61% investigating) and then GPCRs (52% investigating). Least interest was found for CD and organelle markers (**Figure 2**).

Figure 2: Protein classes of greatest interest for pathway analysis
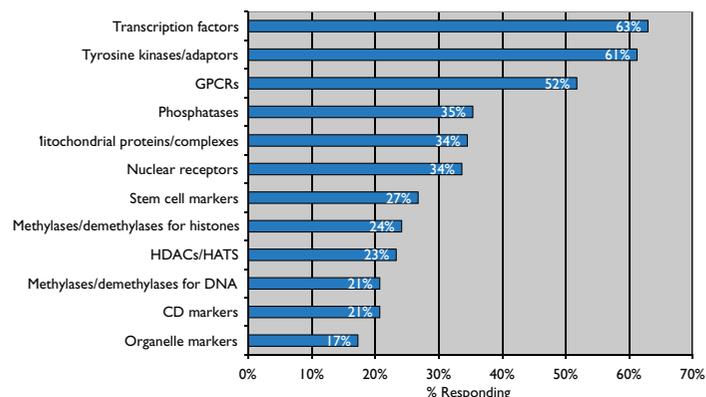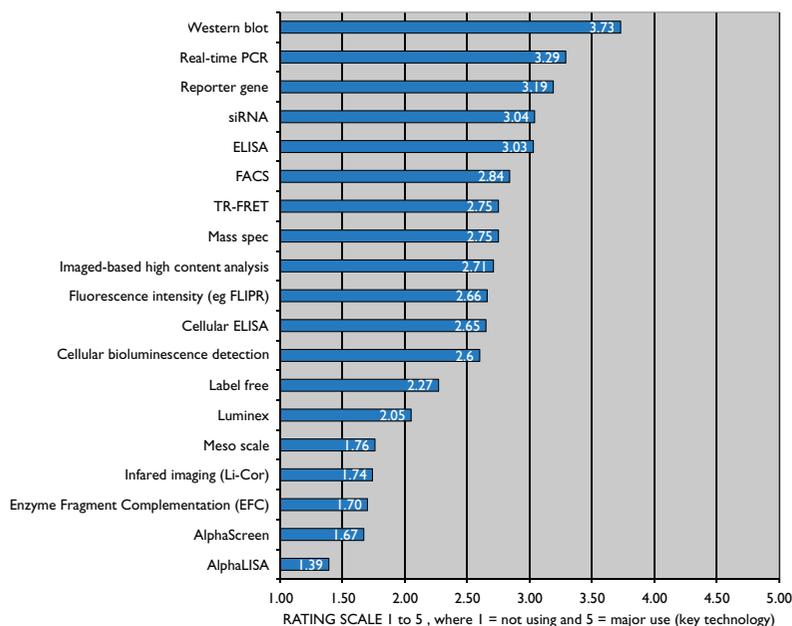

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Figure 3: Signal transduction pathways of greatest interest
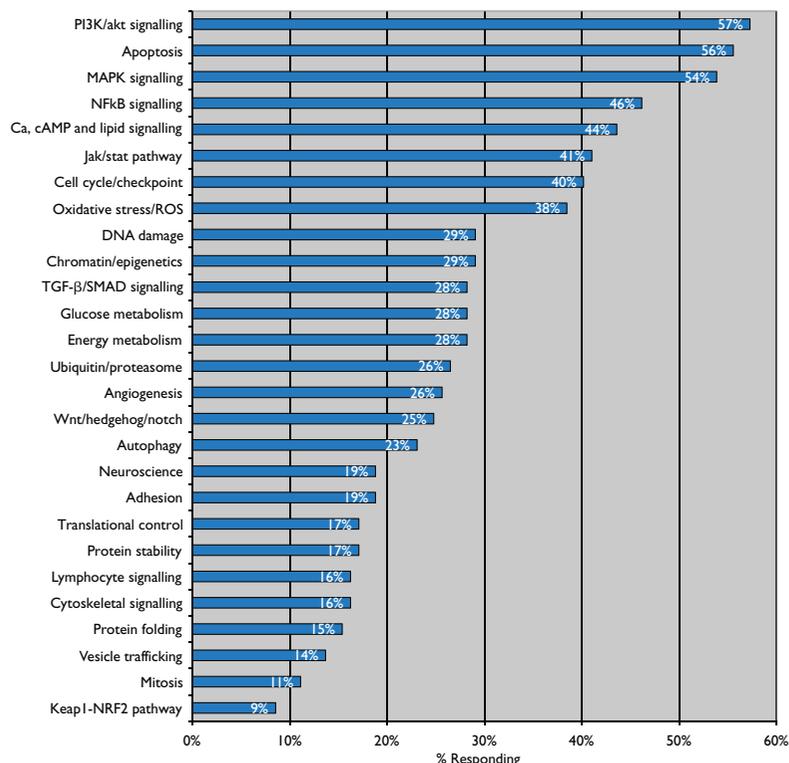
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Pathway Analysis

Figure 4: Main platform technologies used to investigate pathway analysis

Western blot — 3.73
Real-time PCR — 3.29
Reporter gene — 3.19
siRNA — 3.04
ELISA — 3.03
FACS — 2.84
TR-FRET — 2.75
Mass spec — 2.75
Imaged-based high content analysis — 2.71
Fluorescence intensity (eg FLIPR) — 2.66
Cellular ELISA — 2.65
Cellular bioluminescence detection — 2.6
Label free — 2.27
Luminex — 2.05
Meso scale — 1.76
Infared imaging (Li-Cor) — 1.74
Enzyme Fragment Complementation (EFC) — 1.70
AlphaScreen — 1.67
AlphaLISA — 1.39

RATING SCALE 1 to 5 , where 1 = not using and 5 = major use (key technology)

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Figure 5: Stages in drug discovery process investigating pathway analysis assays

Target identification/validation (therapeutic areas) — 78%
Compound profiling — 57%
Assay development/primary screening (HTS) — 50%
Hits-to-leads (lead optimisation) — 44%
Secondary screening — 41%
Preclinical safety/toxicity assessment — 40%

% Responding

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Figure 6: Preferred experimental approach to invesigate pathway analysis

End-point or step specific 18%
By node (nodal points) 14%
By pathway 68%

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Pathways of greatest interest

The signal transduction pathway of greatest interest to survey respondents was PI3K/Akt signalling (57% investigating). This was closely followed by apoptosis (56% investigating); MAPK signalling (54% investigating); and then NFkB signalling (46% investigating). Least investigated were mitosis and the Keap1-NRF2 pathway (**Figure 3**).
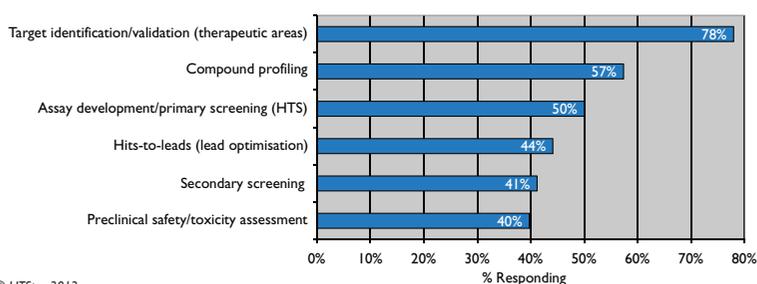
Platform technologies used

Respondents rated western blot as the platform technology they most used to investigate pathway analysis. This was followed by real-time PCR, reporter gene, siRNA and then ELISA. Least used was AlphaScreen and AlphaLISA (**Figure 4**).

The stages of drug discovery investigating

The stage in the drug discovery process most investigating pathway analysis was target identification/validation (ie therapeutic area groups) (78% investigating). This was followed by compound profiling (57% investigating); and then assay development/primary screening (HTS) (50% investigating). Pathway analysis was least investigated in preclinical safety/toxicity assessment (**Figure 5**).

Preferred experimental approaches

The preferred experimental approach to investigate pathway analysis was by pathway (68% preferring); this was followed by end-point or step specific (18% preferring); and then by node (nodal points) (14% preferring) (**Figure 6**).

The preferred experimental approach to investigate pathway measurement was split equally between multiple pathways at a time (eg an array) (51% preferring); versus single pathway at a time (49% preferring) (**Figure 7**).

The preferred experimental approach to pathway annotation was slightly more in favour of broad general pathways (55% preferring); versus into defined groups (eg toxicity etc) (45% preferring (**Figure 8**).
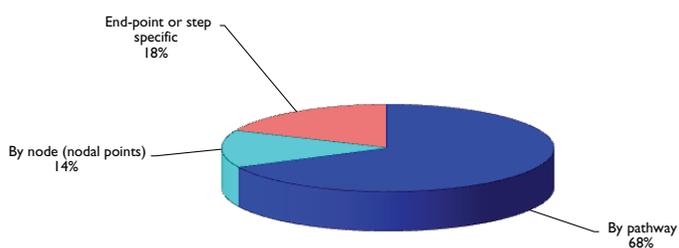
Most important drivers

Survey respondents ranked efficacy as the most important driver for investigating pathway analysis. This was very closely followed by mode of action; then cell-based screening; and toxicity. Ranked least important was drug repurposing (repositioning) (**Figure 9**).

Informatics tools

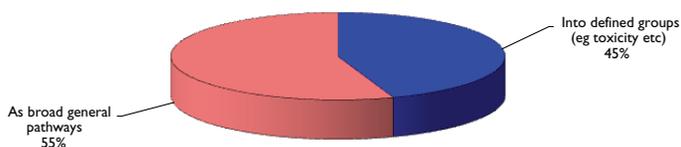Informatics tools are essential to quickly and easily build pathway models and to analyse and

Pathway Analysis

Figure 7: Preferred experimental approach to investigate pathway measurement

Multiple pathways at a time (eg an array) 51%

Single pathway at a time 49%

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Figure 8: Preferred experimental approach to pathway annotation

As broad general pathways 55%

Into defined groups (eg toxicity etc) 45%

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Figure 9: Most important drivers for investigating pathway analysis

	Mean RANKED ORDER
Efficacy	4.64
Mode of action	4.59
Cell-based primary screening	3.55
Toxicity	3.38
Adverse (off-target) events	2.97
Drug repurposing (repositioning)	2.53

Mean RANKED ORDER, where 1 = least important, and 6 = most important

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Figure 10: Informatics tools used to investigate/analyse signaling pathway data

	% Using
In-house developed informatics	73%
Ingenuity	42%
KEGG (pathway solutions)	40%
GeneGo	15%
Other commercial informatics tools	8%
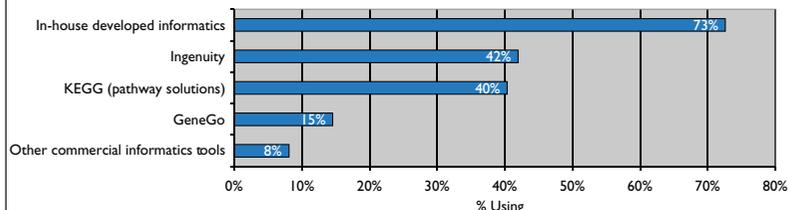
% Using

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interpret experimental data from pathway analysis research. The informatics tool most used to investigate/analyse signalling pathway data was in house informatics (73% using). This was followed by Ingenuity (42% using); Kegg (40% using); and then GeneGo (15% using). Other commercial informatics tools were used also by 8% of respondents (**Figure 10**).

Latest vendor offerings in pathway analysis tools

The MitoSciences® range is exclusive to **Abcam** (www.abcam.com) and is based on advancing mitochondrial studies through the development of novel immunoassays. Product lines include traditional quantitative and profiling ELISAs, specific and relative enzyme activity assays and In-cell ELISA microplate and dipsticks formats. Dipstick assays are a unique and novel technique, which employs lateral flow methods for rapid, simple, sensitive and quantitative analyses. The range also extends to monoclonal antibodies and antibody cocktails with diverse species cross reactivity which are extensively characterised for specificity and sensitivity as well as target-validated by mass spectrometry. MitoTox™ assays are a set of these products for the analysis of mitochondrial drug toxicity. A key control point in cellular metabolism is the mitochondrial pyruvate dehydrogenase complex (PDH or PDC) which links the citric acid cycle and subsequent oxidative phosphorylation with glycolysis and gluconeogenesis, as well as with both lipid and amino acid metabolism pathways. PDH activity is inhibited by site-specific phosphorylation at three sites on the E1 subunit (Ser232, Ser293 and Ser300), which is catalysed by four different pyruvate dehydrogenase kinases (PDK1-4). Analysis of PDH activity in biological samples can be conducted by isolating the active enzyme by immunocapture on to a solid substrate in two ways: 96-well microplate format or lateral flow assays. NADH production can then be measured directly without interference from other NADH-utilising or producing enzymes. Each of the four kinases has a different reactivity for the three phosphorylation sites. Interestingly, phosphorylation at any one site leads to the inhibition of the complex *in vitro*. Two pyruvate dehydrogenase phosphatases (PDP1 and PDP2) dephosphorylate the E1 and activate the enzyme. The four active PDH kinase regulatory enzymes (PDK 1-4) and both PDH phosphatases (PDP 1 & 2) can also be used to activate and inactivate PDH and can be used in both microplate and lateral flow formats. The phosphorylation of E1a serine residues 232, 293,

300 can also be measured in parallel, while the total E1a is simultaneously measured in every well using In-cell ELISA (**Figure 11**).

Bio-Rad (www.bio-rad.com) products are currently being used to detect, characterise and quantify the level of proteins and nucleic acids involved in signalling pathways. From more traditional technologies such as gel electrophoresis and western blotting products to newer technologies such as Digital PCR, ProteOn XPR36 and Bio-Plex multiplex immunoassay system, Bio-Rad products provide a full suite of solutions for researchers in this market space. Bio-Plex, a multiplex immunoassay system based on xMAP bead technology offers cell signalling assays for the simultaneous measurement of multiple phosphorylated proteins in cell culture and tissue samples, from as little as 10ug of sample per 96-well. Designed on magnetic beads, these assays allow researchers to use magnetic separation to simplify assay preparation, decrease variability and increase consistency between experiments. Protein interactions in these pathways can be characterised using the ProteOn XPR36 system, which uses surface plasmon resonance to measure biomolecular interactions on the surface of a sensor chip. Recently launched chips for interaction analysis include the HTG and HTE chips which utilise a tris-NTA surface for histidine-tagged protein capture and the LCP chip for capture of liposomes (**Figure 12**).

Caliper, a PerkinElmer company (www.caliperls. com/CDAS), offers a comprehensive range of contract research service solutions designed to study key disease-related signal transduction pathways. Caliper Discovery Alliances & Services' (CDAS) new offering, called Cell-Based KinaseScreen™, relies on an antibody-based flow cytometry method that quantitatively measures the specific effects of drug candidates on functional inhibition of the kinase targets of interest in a physiologically relevant cellular environment. The technology platform and initial panel of nine assays, including VGEFR, JAK1 and Aurora A, were developed through a strategic scientific collaboration with Pfizer. These assays measure the cellular activity of the kinase of interest by titrating specifically the level of immediate downstream-phosphorylated target protein. CDAS cell-based assays take advantage of multiple fully characterised non-recombinant human cell lines that endogenously express high levels of the kinase target. Use of these relevant cell models provide a more reliable representation of the drug response and greatly reduces the
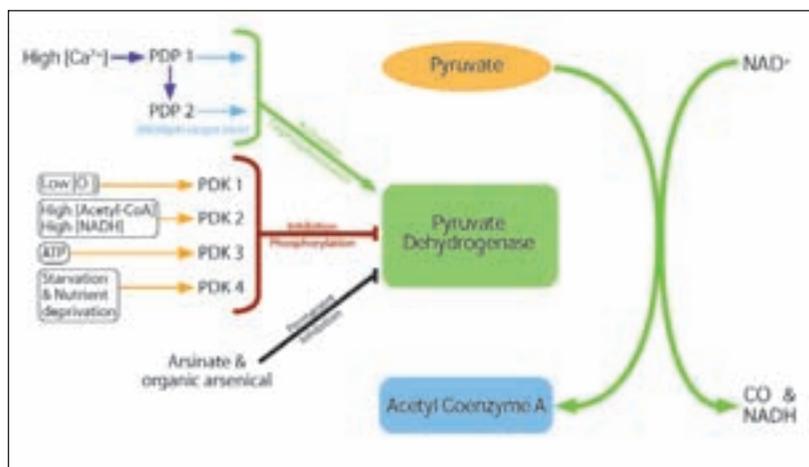

Figure 11: Abcam's MitoTox assays for mitochondrial toxicity analysis. The activity of PDH is regulated by reversible phosphorylation of three serine residues on the E1alpha subunit. The phosphorylation of these sites is catalysed by PDH kinases (PDK). Dephosporylation to restore the activity of PDH is catalysed by PDH phosphatases (PDP). There are two known isoforms of PDPs, which are expressed differently in various tissues. Each of the PDK's and PDP's is under transcriptional control in response to different cellular stress events as shown. In addition, the kinases are activated by acetyl coenzyme A, NADH and ATP, meanwhile the availability of pyruvate and ADP leads to their inhibition

chances of false positive/negative data. These assays are used early in the drug discovery process to assess compound selectivity, and evaluate potential 'off-target' effects of kinase-controlled pathways. Multiple levels of assay specificity considered in developing these assays are important for

Figure 12: Bio-Rad's New V3 workflow incorporates products from electrophoresis and blotting to imaging for Pathway analysis

Pathway Analysis

Figure 13: Multiple levels of specificity in Caliper's CDAS Cell-Based KinaseScreen assays include: 1) specific pathway stimulation using selective inducers; 2) specific target kinase activity; and 3) specific epitope-targeted phosphorylation. This allows to distinguish between different upstream effectors and to specifically study the pathway of interest

the development of safer drug candidates and the reduction of late-stage drug attrition (**Figure 13**). CDAS' growing offering of kinase functional serv-

ices will include PerkinElmer cell-based AlphaScreen®SureFire® assays to analyse pathways, screen GPCRs, growth factor receptors, and characterise intracellular kinase inhibitors of main signalling pathways.

Corning (www.corning.com/lifesciences/epic/en/index.aspx) approaches pathway analysis with the unique perspective of analysing signalling activity in endogenous pathways within cells, without the need for fixation, labelling or perturbation of the cells using over-expressed, tagged proteins. Using the label-free technology available on the Epic® instrument platform, it is able to monitor cellular activity in virtually any signalling cascade, and deconvolute the contribution of various downstream signalling nodes using available tool compounds. Corning has addressed a variety of target classes (GPCRs, kinases, ion channels, cytokine receptors, etc) in a range of cell backgrounds, including complex systems such as primary cells and different types of adult stem cells. Furthermore, it has recently made this technology
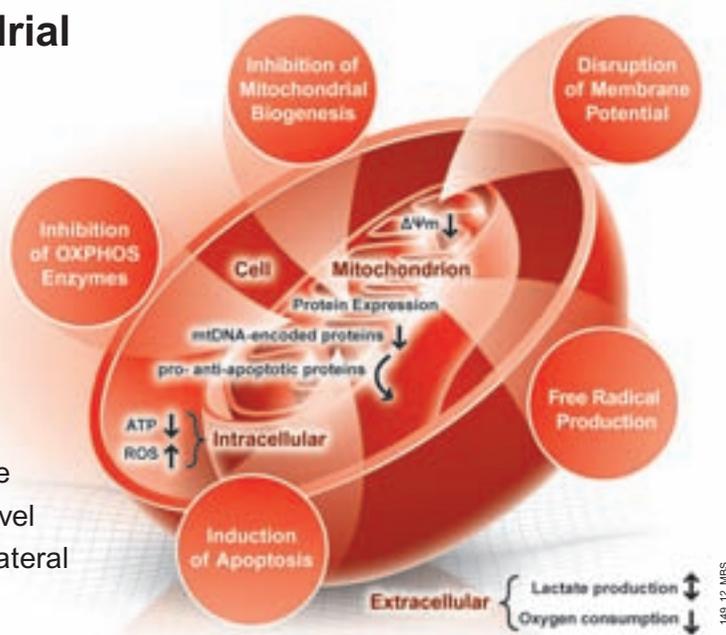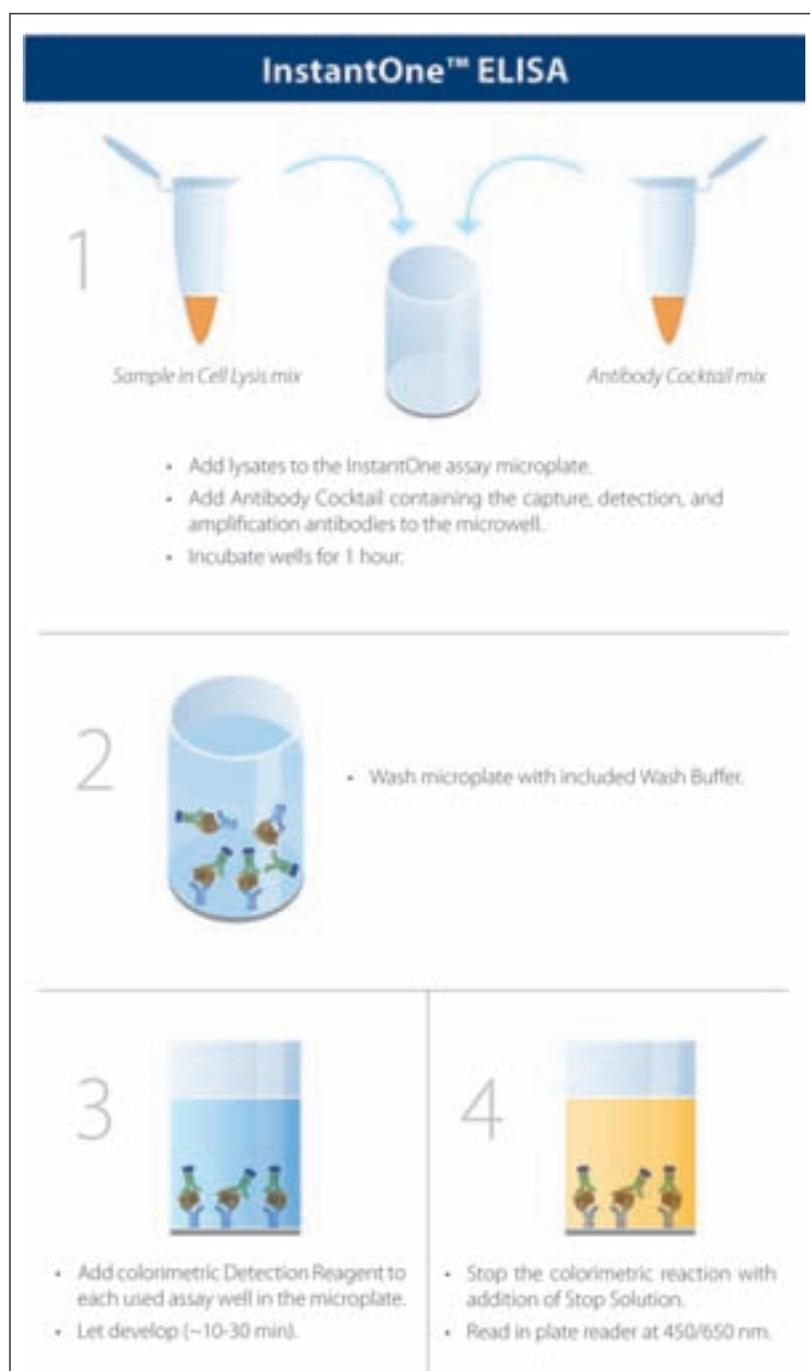
accessible to the general public through its Epic® Discovery Service business, offering contract screening, assay development and most recently cellular profiling services. Using its unique set of assay approaches, Corning has been able to answer complex pharmacological challenges, including identification of biased ligand signalling and deconvolution of downstream signalling, prediction of adverse and off-target effects for late stage compounds, and even elucidation of mechanism of action for uncharacterised off-target effects. Given the unique nature of this technology, and its ability to analyse cellular events in a target agnostic fashion, Corning also believes this can provide an ideal platform for drug repurposing projects, and hopes it will be able to extend its utility to prediction of compound toxicity in the very near future.

The InstantOne™ cell signalling assays from **eBioscience** (www.ebioscience.com) enable more efficient phosphorylation profiling and target validation data than western blot or traditional ELISAs. InstantOne assays require only one wash step, take 75% less time than traditional phospho-ELISAs and provide the flexibility to perform either single or multi-target analysis on the same plate. Whether you are studying phosphorylated protein levels, profiles across families or entire pathways, the InstantOne ELISA is the solution. These performance advantages are enabled by vastly improved antibody-to-target affinity and in-solution target capture prior to solid phase. The InstantOne ELISA has proven to be both easier and more sensitive that many traditional ELISAs and homogeneous TR-FRET assays with no additional equipment needed in most cases. The InstantOne ELISAs run on traditional colorimetric plate readers and are available in either 96-well or 384-well format. (**Figure 14**).

Challenges facing signalling pathway elucidation include accurately modelling redundancy, feedback, crosstalk and cell-extrinsic/cell type-specific signalling. These challenges require a multidisciplinary, multianalyte, cross-platform approach. In response, **EMD Millipore** (www.millipore.com/drugdiscovery) has developed a number of synergistic platforms and technologies for signalling research. First, MILLIPLEX® MAP EpiQuant technology can quantify up to 40 analytes, including site-specific phosphorylation events, in a single sample, using bead-based multiplexed detection on the Luminex® platform. While traditional signalling assays can only reveal relative changes in protein levels, EpiQuant™ assays provide absolute quantification of total and phosphorylated pro-

teins, enabling direct comparison of events in different pathways. Second, EMD Millipore's new Amnis® imaging flow cytometers enable automatic, rapid image analysis in suspension. Numerically scoring large numbers of automatically acquired, multicolour images is ideal for analysing signal transduction in heterogeneous cell populations and correlating signalling events with phenotypes. Third, EMD Millipore's new InhibitorSelect™ libraries and signalling pathway panels enable the

Figure 14: Schematic of eBioscience's InstantOne ELISAs for cell signaling assays
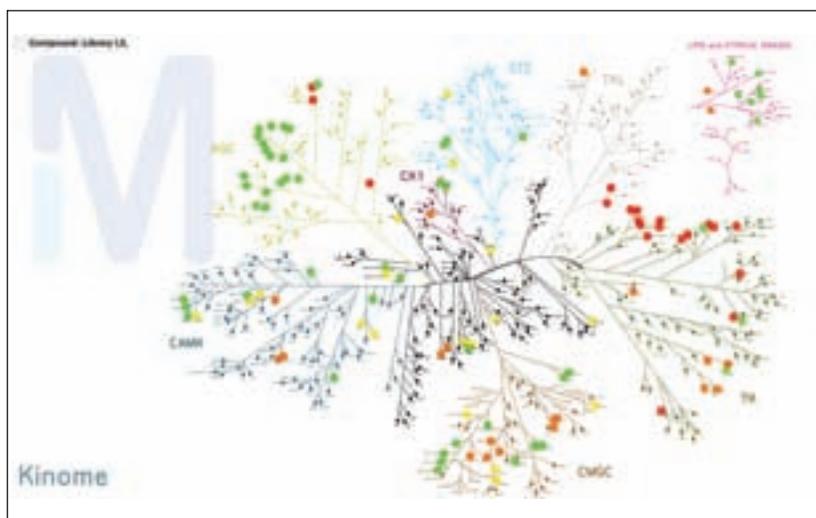
Pathway Analysis

Figure 15: A selectivity profile of a specific inhibitor from the InhibitorSelect™ kinase inhibitor libraries is visually displayed on the kinome map using EMD Millipore's data analysis and report tool (DART™). By creating interactive maps of KinaseProfiler™ service results, the DART™ tool enables faster, more informed decisions about the effects of drug candidates on signalling proteins

interrogation of signalling via chemical genetics. Compared to traditional genetics, small molecules inhibit quickly and reversibly, providing dose response information with spatiotemporal resolution. The InhibitorSelect™ pathway panels inhibit multiple proteins along a specified pathway, revealing signal amplification, feedback and relative effects of perturbing different parts of the pathway. Finally, EMD Millipore's new SignalProfiler™ service profiles drug compounds across many different signalling pathways and can indicate whether signalling feedback or cross-talk is interfering with a
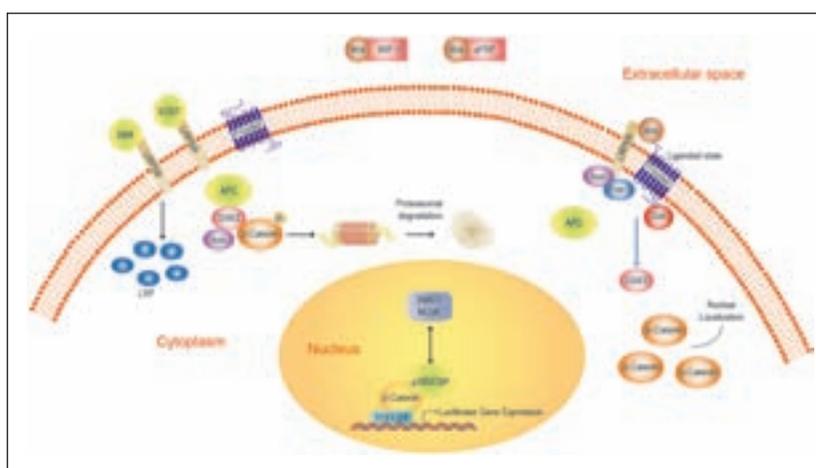

Figure 16: Schematic of the Wnt signalling pathway and its components. Luciferase activity from the reporter gene in Enzo's Leading Light™ Wnt cell line can be up- or down-regulated in a dose-dependent manner upon the addition of exogenous Wnt protein, Wnt agonist or Wnt antagonist (DKK) to the cell culture medium

compound's effectiveness. The first such service to use therapeutically relevant cells, SignalProfiler™ uses trusted Luminex® technology to provide deep, predictive insights into the biological efficacy and safety of compounds (**Figure 15**).

Wnt signalling plays important roles relating to development and disease. **Enzo Life Sciences'** (www.enzolifesciences.com) Leading Light™ Wnt Reporter Assay contains an engineered 3T3 mouse fibroblast cell line with a luciferase reporter gene under the control of Wnt-responsive elements (TCF/LEF). The luciferase activity can be regulated in a dose-dependent manner by addition of exogenous Wnt protein, Wnt agonist or Wnt antagonist to the cell culture medium. The microplate assay detects low concentrations of effectors using a short six-hour protocol and Wnt3a stimulation produces up to a 35-fold induction of signal over baseline. The assay generates a high signal-to-noise ratio and Z'-factor score. It is useful for analysis of the functions and activities of different Wnt-related ligands such as DKK, R-spondin and sFRP. It is also suitable for screening small molecules and antibodies as Wnt inhibitors or Wnt agonists. Enzo's Sclerostin-LRP Binding Assay is a microplate-based biochemical assay whereby LRP5-alkaline phosphatase can interact with immobilised sclerostin. This interaction is measured by the activity of alkaline phosphatase in a 96-well format, generating high signal-to-noise ratio and Z'-factor score. The assay is suitable for screening small molecules, antibodies and peptides, which can block sclerostin-LRP interaction. Enzo's Screen-Well® Wnt Pathway Library contains 75 validated compounds, many of which are proprietary to Enzo, with diverse Wnt pathway activity, including activators and/or inhibitors of Wnt, Dishevelled, GSK-3ß,TCF/ß-catenin, DKK, LRP, Axin and Porcupine. Additionally, Enzo offers GSK-3ß, DKK-1 and ß-Catenin ELISA kits as well as purified proteins and antibodies relating to the Wnt signalling pathway (**Figure 16**).

IPA® from **Ingenuity** (www.ingenuity.com) is a web-based software application that enables life science researchers to analyse, integrate and understand data derived from gene expression. IPA supports microRNA and SNP microarrays; metabolomics, proteomics and RNA-Seq experiments; and small-scale experiments that generate gene and chemical lists. Researchers can analyse experimental data in the context of known biology to quickly narrow in on what is most important in their datasets and discover novel relationships. IPA delivers a rapid

Pathway Analysis

Figure 17: Antigen presentation pathway modelled using Ingenuity's IPA web-based software application

assessment of the signalling and metabolic pathways, molecular networks and biological processes that are most significantly perturbed in the dataset of interest. The newest release of IPA helps researchers better understand the cause and effect of gene expression changes in their experiment. New tools enable researchers to predict which transcription factors could be responsible for gene expression and whether those transcription factors are activated or inhibited, all based on experimentally observed relationships. It is also possible to visualise downstream effects, to determine if differentially

Figure 18: Schematic of Lifetech's LanthaScreen® cellular assay platform. Cells are mixed with BacMam Reagent encoding GFP-tagged Histone H3 protein and plated on to a 384-well assay plate. Cells are left untreated or treated with compound for 20 to 24 hours. Cells are lysed in the presence of a terbium (Tb)-labelled modification-specific antibody and TR-FRET is detected using a fluorescence microplate reader with standard TR-FRET settings

expressed genes are causing an increase or decrease in downstream biological processes or diseases. Researchers can also understand isoform-specific biology with an integrated, interactive view of each human locus that supports the exploration of each locus, its spliced mRNA and protein domains. Pathway solutions require a large database of high quality information to enable their capabilities. IPA is powered by the Ingenuity® Knowledge Base, which includes modelled relationships between proteins, genes, complexes, cells, tissues, drugs, pathways and diseases. It also houses information from a broad range of published biomedical literature, internally curated knowledge, and a wide variety of trusted 3rd party sources and databases, so researchers can access a wide variety of information in one place (**Figure 17**).

Life Technologies (www.lifetech.com) offers two assay platforms and rapid profiling services for high-throughput pathway analysis. One platform, CellSensor® pathway assays, uses GeneBLAzer® beta-lactamase reporter gene technology to provide a rapid and sensitive method of analysing endogenous signalling pathways in response to environmental cues. Broad pathway coverage of these assays enables a multi-pathway cellular profiling approach that can be used to generate compound pathway-selectivity profiles, efficiently characterise both on and off-pathway compound activities and reveal potential novel pathways and opportunities for drug repositioning purposes and/or safety liabilities in one profiling campaign. A second assay platform, LanthaScreen® cellular assays, is ideal for analysing a specific signalling event within a pathway in response to environmental cues. These assays utilise cells expressing green fluorescence protein (GFP)-substrate fusion protein stably engineered or transiently delivered via BacMam and terbium labelled-anti-modification-specific antibodies. The simple addition-only workflow enables the application of these assays in the cellular detection of protein phosphorylation, acetylation, ubiquitination and histone methylations and represents a novel high-throughput alternative to commonly-used western blot analysis and ELISA type of assays. For labs needing additional resources to conduct pathway profiling, the SelectScreen® cell-based pathway profiling service utilises CellSensor® pathway assays to offer high-quality data and rapid turnaround times – enabling researchers to understand potential off-target effects faster, while giving your lab team time to focus on other priorities. LanthaScreen® cellular assays are also available for pathway profiling services on a custom basis (**Figure 18**).

MBL International (www.mblintl.com) offers several solutions for the discovery of RNA signalling pathways. Its RIP-Assay Kit and RiboTrap Kits allows researchers to navigate the discovery of RNA binding proteins (RBPs) and their targets. The RiboTrap Kit is used to isolate RBPs and other proteins associated with mRNA, miRNA and other RNA from cytoplasmic or nuclear extracts of mammalian cells. This kit provides an immunoaffinity method to explore RNA-protein and RNA-RNA interactions. RiboTrap illustrates the endogenous assembly of ribonucleoproteins and identifies regulatory components in the biosynthesis of mRNA, small RNA and non-coding RNA. The RIP-Assay Kit utilises a technique named RIP-Chip (ribonucleoprotein immunoprecipitation-microarray profiling) which is a biochemical approach to identify the composition and organisation of endogenous mRNAs, miRNAs and RNA binding proteins (RBPs) within messenger ribonucleoprotein (mRNP) complexes. When the co-isolated miRNA and mRNA subpopulations are analysed using the computational predictions of conserved seed sequences, this approach provides a powerful tool to identify functional miRNA targets based on their physical interaction *in vivo*. RBPs have been reported to bind to mRNAs that encode functionally related proteins and co-ordinate regulation of these mRNAs during cellular processes. The RIP-Chip approach can isolate functionally-related mRNAs. Since miRNAs can be co-immunoprecipitated with those mRNAs, the RIP-Chip approach can isolate miRNAs that regulate specific groups of mRNAs that are functionally related. Both the RiboTrap Kit and RIP-Assay Kit allows for the discovery of the RNA protein network. By offering an immunoaffinity method of exploring RNA-protein and RNA-RNA interactions, researchers now have a fully biological approach to finding functionally related genes of diseases pathways (**Figure 19**).

The latest advancements in imaging and analysis technologies from **Molecular Devices** (www.molec-ulardevices.com) are being used by bioresearchers globally to accelerate their understanding of cell signalling pathways. Version 2 of the MetaMorph® NX Microscopy Automation and Image Analysis Software takes the learning curve out of custom pathway analysis through the use of visual, step-by-step guided assistance for designing customised, distributable and reusable image processing and analysis modules. Utilising the Custom Measurement feature, you no longer need programming experience to easily automate multifac-
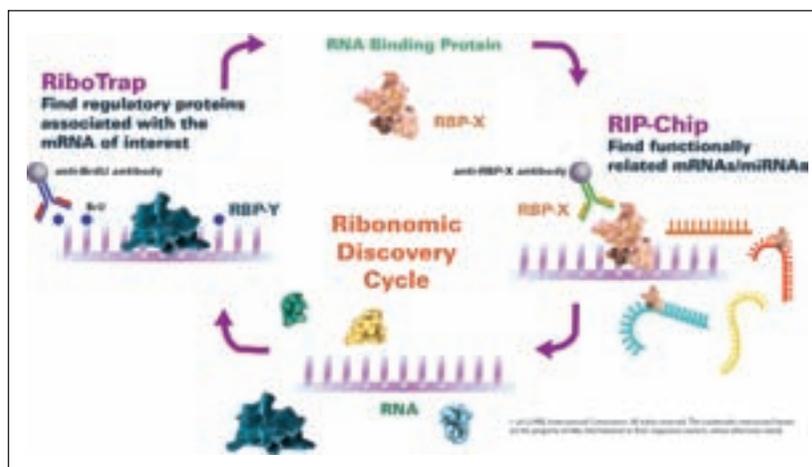

Figure 19: MBL International RiboTrap and RIP-Chip kits for the discovery of RNA signalling pathways

eted assays of datasets. Graphical tools reduce iteration cycles by creating, adjusting and viewing the results of your analysis all at the same time. Pre-processing steps are now easily combined with segmentation and measurements to extract features, increase the level of detail and remove unwanted image artifacts. The ImageXpress® Micro XL Wide Field High Content Screening System uses state-of-the-art detector and illumination technologies to capture cellular resolution images in a single field encompassing one 384-well plate. Combined, these technologies increase content to three times more than what is acquired with standard HCS systems. Assay window and reliability are enhanced with 3-log dynamic range and less than 5% CV for intensities across the plate. The accompanying software, version 4 of the MetaXpress® Software, increases acquisition and image analysis to more than 10 million cells per hour. Combined, these new imaging and analysis technologies simplify, expedite and increase the throughput of numerous pathway analyses including: receptor desensitisation, stem

Figure 20: New imaging and analysis technologies from Molecular Devices: A) the new custom measurement (analysis) workspace in version 2 of the MetaMorph NX software; and B) the base unit of the ImageXpress Micro XL System

cell differentiation, inflammatory response, host signalling response to infectious agents, cancer biology and neurogenesis (**Figure 20**).

Pathway Studio® from **Elsevier** (www.pathwaystudio.com) is a biological informatics system that integrates millions of literature-derived facts in a data mining interface for pharmaceutical research. Elsevier applies proprietary text-mining technology to scientific literature to extract facts about molecular biology, disease and chemistry in order to produce high-quality structured knowledge bases for analysis in Pathway Studio. Using Pathway Studio, researchers can access facts about indirect relationships and directly observed connections between hormones, cell receptors and transcription factors to understand signal transduction. Pathway Studio complements an experimental approach with hypothesis generation tools and by providing algorithms that analyse multiple channels of 'omics information. Pathway Studio has been used to validate and confirm research in many different disciplines. Wyeth researchers demonstrated a workflow using Pathway Studio to identify the effectiveness of compounds in blocking signal transduction based on phosphorylation and proteomics assays. In 2010, Cellecta and Roswell Park Cancer Institute used Pathway Studio to develop a panel of candidate prostate cancer survival genes and subsequently to interpret the prostate cancer cell line screening experiments which identified genes essential for viability of prostate cancer cells in order to reveal novel targets and biomarkers. Most recently, researchers at Sanofi-Aventis utilised Pathway Studio to develop a panel of indicators that could be used to differentiate various subtypes of lung cancer. Pathway Studio includes expertly curated reference pathways that researchers can use to assemble large signal transduction maps such as one produced by biologists at Elsevier for publication, which combines accepted mechanisms for prostate cancer growth and therapy into a single network (**Figure 21**).

Promega (www.promega.com) has developed a novel bioassay for Fc effector function that leads to antibody-dependent cell-mediated cytotoxicity (ADCC). It incorporates an NFAT response element upstream of a firefly luciferase gene. The bioassay exploits activation of the NFAT signalling pathway that occurs naturally during ADCC mechanism of action in NK cells, following cross linking of FcgRIIIa receptor with target cell-bound specific antibody. A Jurkat cell line engineered to contain both the FcgRIIIa receptor and the luciferase reporter construct serves as the universal effector
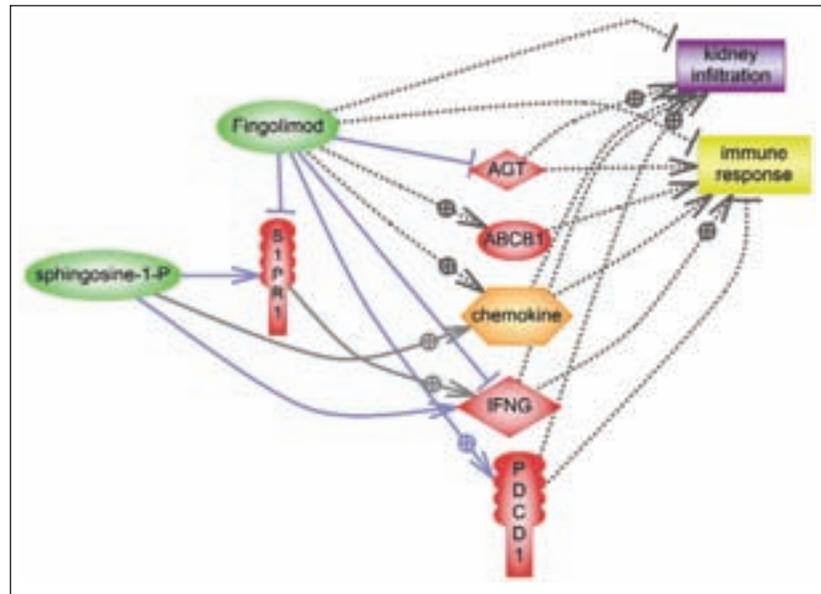

Figure 21: Pathway Studio from Elsevier provides an overview summary for Fingolimod (Gilenya™), a drug approved for Multiple Sclerosis, and tested in clinical trials for immune response suppression in organ transplant. Solid lines show direct observations regarding action of Fingolimod through S1PR1 to inhibiting the transport of Sphingosine-1 Phosphate and action on key constituents. Dashed lines show indirect observations of inhibition of Immune Response and Kidney Infiltration. Additional modelling effort can replace indirect observations with direct molecular relationships to elucidate mechanism of action

cell population. When combined with biologic antibody drugs and relevant target cells expressing the appropriate antigen for the biologic drug, ADCC mechanism of action pathway activation occurs; readout is from the effector cell population. Classic ADCC assays rely on primary NK cells (from blood) and cytotoxicity of the target cells as readout. Such assays suffer from high variability, and are complicated and tedious to perform. Promega's reporter-based approach with an engineered effector cell line demonstrates the requirements for an
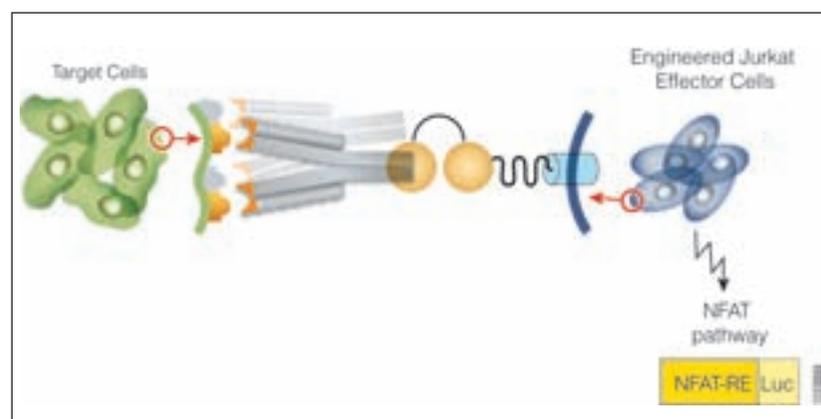

Figure 22: Promega's new reporter bioassay exploits activation of the NFAT signalling pathway as readout for ADCC
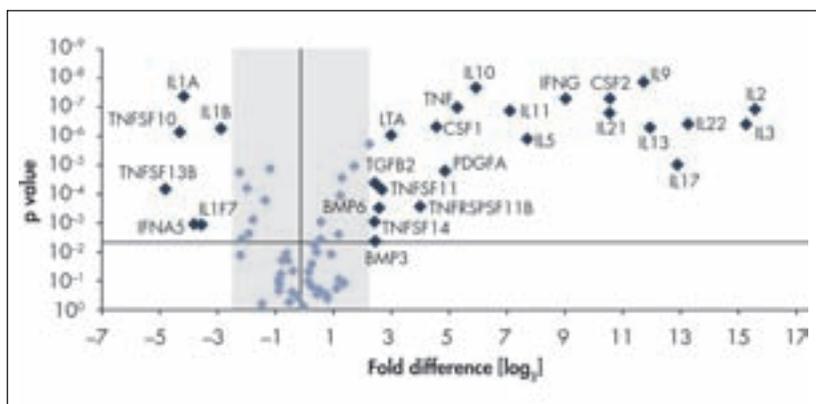
Pathway Analysis

Figure 23: Common Cytokine RT2 Profiler PCR Array from QIAGEN identified 23 up-regulated and six down-regulated genes following PBMC stimulation. Triplicate total RNA samples from human peripheral blood mononuclear cells (either untreated or stimulated with 50ng/ml PMA and 1mg/ml ionomycin for six hours) were characterised with the human Common Cytokine RT2 Profiler PCR Array. Twenty-three cytokine genes are up-regulated (> 5-fold, p < 0.0005) including interleukins, colony stimulating factors, and TNF ligands after six hours of stimulation. Six interleukin and TNF ligand genes are down-regulated under the same conditions

appropriate bioassay: stability-indicating, good accuracy, precision, reproducibility and linearity across potencies. Importantly, it is able to quantify Fc effector function of therapeutic antibodies in ADCC mechanism of action, including differentiating antibodies with small differences in glycosylation. The reporter-based ADCC bioassay is shown in **Figure 22**.
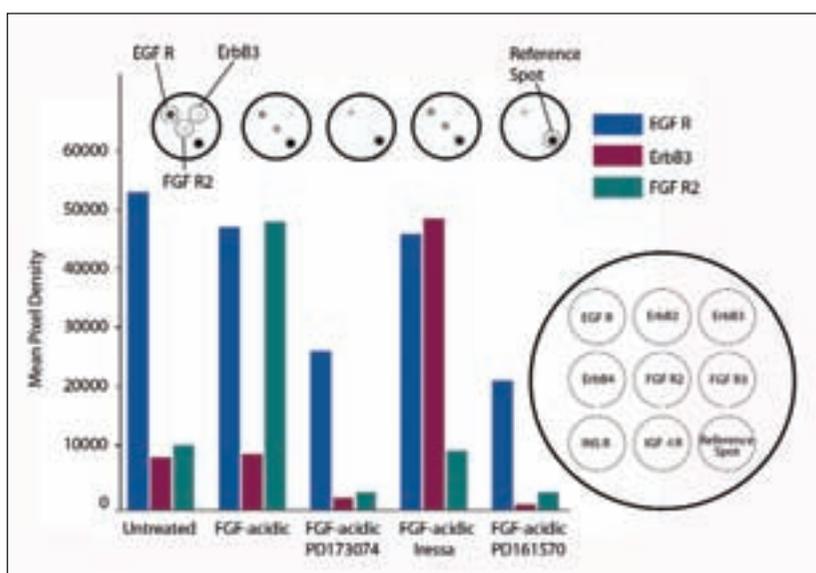

Figure 24: Induction and inhibition of receptor tyrosine kinase phosphorylation in gastric cancer cells. Well images from an R&D Systems Proteome Profiler 96™ Phospho-RTK Custom Array and the corresponding histogram profiles are shown with the array layout. KatoIII cells were untreated or treated with RTK inhibitors followed by treatment with FGF acidic and heparin. The array was used to monitor the effects of inhibitors on kinase phosphorylation in KatoIII cells

QIAGEN (www.qiagen.com) offers pathway-focused profiling for gene and miRNA expression and DNA mutation and methylation status. Understanding the function of a protein in the context of normal and abnormal cellular processes requires a comprehensive knowledge not only of its regulation but also of its role in signalling and metabolic networks in the cell. A molecular understanding of complex diseases, and the goal of developing targeted therapies against them, requires a deep dive not just in gene expression analysis, but also in the identification and characterisation of epigenetic modifications or somatic mutations that may contribute to disease progression. The ability to perform such detailed analysis of gene networks was previously the exclusive domain of labs with access to advanced robotic screening and bioinformatics resources. QIAGEN offers all the tools, from sample preparation through data analysis and interpretation, to take your research to a higher level of sophistication, no matter the scale of your research project. Best-in-class bioinformatics for gene content is coupled to innovative assay design, extensive wet lab validation, dedicated and optimised chemistry for all major PCR platforms, and dedicated free on-line data analysis tools. This tightly integrated system leverages your real-time PCR platform into a robust turn-key solution for systems biology research (**Figure 23**).

Proteome Profiler 96™ Arrays from **R&D Systems** (www.RnDSystems.com/ProteomeProfiler96) are used to simultaneously measure relative changes in the phosphorylation of multiple receptor tyrosine kinases (RTKs) and cytoplasmic kinases involved in signal transduction. The array is capable of detecting changes in the phosphorylation of up to 16 kinases in each well of a 96-well plate for a total of 1536 possible data points per plate. This format also offers the advantage of a small sample size requirement, and data collection with chemiluminescence or near-infrared imagers commonly used for western blot. It is available either as a pre-defined array or in a custom format that allows researchers to design their own assay. In this experiment, 8 RTKs were selected from a panel of 40 and custom printed in the bottom of 96-well plates. To measure the effects of inhibitors on kinase phosphorylation, cells were untreated or treated with 40nM of the RTK inhibitors PD173074, Iressa or PD161570 (Tocris) for three hours. This was followed by treatment with 100ng/mL of recombinant human FGF-acidic (R&D Systems) and 1 g/mL of heparin for 15 minutes. KATOIII cells overexpress

FGF R2 and an increase in FGF R2 phosphoryla-tion was observed following FGF stimulation. The FGF R-specific inhibitors PD173074 and PD161570 were shown to suppress FGF R2 phos-phorylation. Because KATOIII cells are Iressa resistant, this EGF R inhibitor does not affect EGF R phosphorylation levels (**Figure 24**).

Sigma Life Science (www.sigma-aldrich.com) is applying its powerful CompoZr® zinc finger nuclease (ZFN) technology to create immortalised cancer cell lines having key signalling pathway and cytoskeletal genes tagged with fluorescent reporter proteins. A distinct advantage of this technology is the ability to make fusion proteins from the endogenous loci of these genes such that expression remains under control of the endoge-nous promoter yielding native levels of the protein within the cell. This avoids artifacts associated with protein overexpression on dynamic biologi-cal processes and permits real time, live cell imag-ing to study protein localisation and subcellular translocation in response to various stimuli such as growth factors, cytokines and/or drug treat-ments. **Figure 25** illustrates the cytoplasmic to nuclear translocation of STAT3 in a human ovari-an carcinoma cell line, SKOV3 GFP-STAT3, with-in 10-30 minutes of stimulation by 100ng/mL IL-6. These cell lines provide a novel tool for exam-ining basic biology or for high-content analysis in drug screening programmes. Notably, the technol-ogy is amenable to tagging multiple genes within the same cell line to study the complex dynamics between individual genes such as demonstrated for clathrin-mediated endocytosis[2]. Sigma is now extending this gene-tagging technology to human inducible pluripotent stem (iPS) cells where it can be applied, among other things, as a marker to track differentiation into a specific cell lineage. Tagging endogenous gene loci in these cell lines is critical for studying native biological pathways and processes (**Figure 25**).
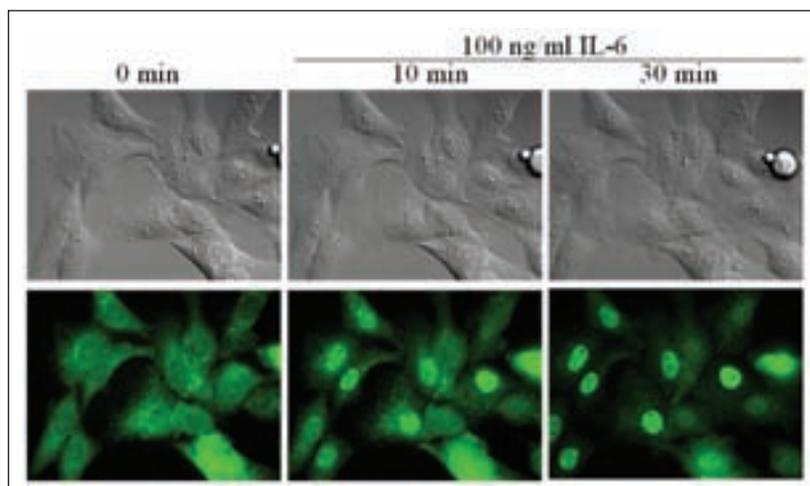

Figure 25: IL-6-induced nuclear translocation of endogenously-tagged GFP-STAT3 in human SKOV3 cells. The human ovarian adenocarcinoma cell line, SKOV3, was modified with ZFN technology from Sigma Life Science to create a GFP-STAT3 fusion protein made from the endogenous gene locus. The cells were treated with 100ng/mL IL-6 which stimulated cytoplasmic to nuclear translocation of STAT3 (green fluorescence) within 10-30 minutes

Figure 26: The heat map shows the inducible activity of 48 different reporter constructs (rows) in 15 different conditions (columns). The reporters are a combination of endogenous human promoters and synthetic response elements cloned into SwitchGear Genomics LightSwitch vectors. Promoters that are upregulated are shown in red and downregulated promoters are shown in blue. Treatments were done with known pathway inducers (eg DFO and 1% O2 strongly induce the hypoxia pathway). Some pathways are activated by multiple conditions (eg PMA and TNF both strongly activate the NFkB reporters. All experiments were performed in HT1080 cells
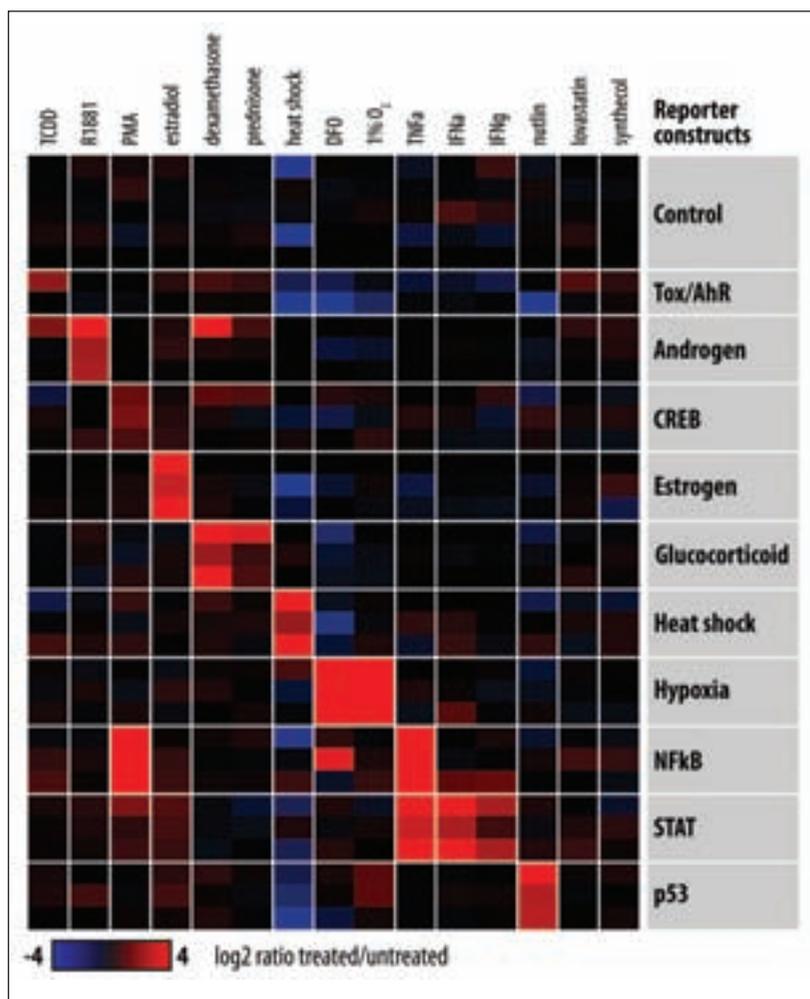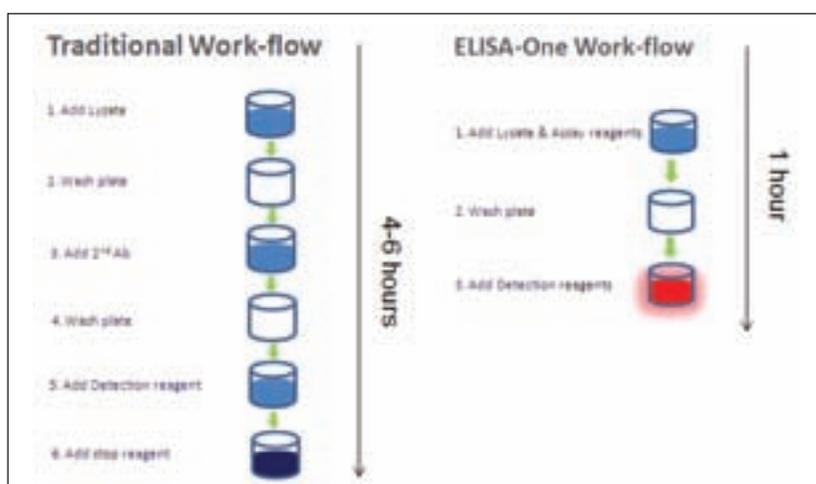
Pathway Analysis

Figure 27: TGR BioSciences ELISAONE™ assay protocol for cellular phosphoprotein detection. The new ELISAONE™ assay system from TGR is rapid (one hour) and has a single wash step, unlike standard ELISAs. Further, an important attribute of the system is that any target can be measured in any well, since both antibodies are added in solution rather than one pre-bound to the assay plate. This provides great flexibility to the researcher, with every well in the plate potentially measuring a different target from the complete list of kits from TGR

The LightSwitch Luciferase Assay System™, developed by **SwitchGear Genomics** (www.switchgeargenomics.com), is a full reporter assay solution. The LightSwitch System, a powerful platform for measuring changes in gene expression in a cell-based system, includes pre-cloned, genome-wide collections of human promoter and 3'UTR reporter constructs, synthetic response elements, and optimised luciferase assay reagents. The LightSwitch System also includes a unique collection of experimentally-validated, fully characterised reporter assays that 1) eliminates the need for end-user assay development and 2) can readily be used in screening applications to measure the effects of compounds on a variety of biological pathways. Pathway-specific assays were validated for a variety of pathways including Tox/AhR, CREB, androgen, estrogen, glucocorticoid, heat shock, hypoxia, NFkB, STAT, SREBP and p53. The LightSwitch System is a cost-effective and high-throughput platform for primary and secondary screening, off-target analysis, lead compound validation, and dose-response analysis. The system includes comprehensive and streamlined protocols that support the customer's entire workflow from cell seeding to data collection. Changes in promoter activity result in changes in luciferase expression. The resulting luminescent readout of the assay indicates biological pathway activity based on the validated LightSwitch promoter assay. SwitchGear Genomics offers both a complete service option where researchers can outsource the

screening of up to 1,000 compounds and the option to screen compounds in a researcher's own lab using the company's comprehensive product kits (**Figure 26**).

TGR BioSciences (www.tgrbio.com) specialises in state-of-the art assays for cellular pathway analysis. TGR has two main product offerings, directed toward the HTS marketplace as well as those with lower sample number requirements and more standard assay equipment. To address the HTS market, TGR developed the AlphaScreen® SureFire® assay portfolio, measuring endogenous phosphoproteins in cellular lysates. Used in combination with the highly sensitive Alpha Technology, SureFire® assay kits enable examination of various signalling pathways, and receptor activation, in a native and endogenous protein cell-based format. The high throughput aspect of this technology is due to there being no wash steps. It is a truly mix-and-read plate-based assay system, with the high sensitivity required for native cellular protein detection. TGR markets these products exclusively with PerkinElmer. AlphaScreen® SureFire® assays require an Alpha-compatible plate reader. For those groups also measuring cellular signal transduction, but with standard plate reader technology only, TGR has also developed a new ELISA technology that is both faster and easier to carry out than standard ELISAs. This is a single-wash, one-hour assay system, called ELISAONE™ which delivers a highly sensitive assay without the tedium associated with standard ELISAs. The ELISAONE™ kit portfolio has the major cellular phosphoprotein targets covered, including ERK, AKT, NF-kB, STAT proteins and others. Assays developed on TGR's ELISA platform are available with either fluorescent or colorimetric detection, from a number of vendors (**Figure 27**).

Over the last decade, **Thomson Reuters** (www.genego.com) has developed an original 'knowledge-based' methodology of functional analysis of OMICs data, gene/protein lists and small molecule compounds of arbitrary structure. The approach consists of creating a comprehensive, manually curated database of protein interactions (more than 1 million), pathways and biomarkers from experimental literature and using these structured data by a set of 'systems biology' tools for ontology enrichment, interactome and network analysis. The database semantics and architecture allows coupling of signalling interactions, metabolic and transport reactions, which enables seamless integration and concurrent analysis of multiple

types of data, for instance gene expression, genetic variants, protein and metabolic profiles. The database-rich semantics includes eight original ontologies including pathway maps, process and diseases networks, disease biomarkers, animal toxicities; controlled vocabularies and a synonyms dictionary of more than seven million terms. As both protein interactions and gene disease associations are annotated, the pathways can be visualised in a 'mechanistic' and 'causal' modes and the phenotypes (ie disease subtypes, toxic end-points, drug response etc) can be represented as a 'barcode' of contributing normal and signalling pathways. These pathway profiles can be applied quantitatively for drug repositioning, personalised and translational medicine. The 'knowledge base' is realised through several web-based products: MetaCore for OMICs data analysis, MetaDrug for prediction of biological effects of drug-like compounds, add-on modules for toxicity, stem cells and common diseases. In addition to off-the-shelf product offering, Thomson Reuters professional services team is engaged in a variety of custom projects, ranging from OMICs data analysis and causal network modelling for patient stratification to semantic-based projects such as development of assay registration systems, annotation of clinical data and drug repositioning (**Figure 28**).

Discussion

From the vendor snapshots it is possible to pull together the following trends in new tools for signalling pathway analysis:

ELISA: New ELISA methods are proving very useful in studying phosphorylated protein levels, profiles across families or entire pathways. Cellular ELISAs requiring less time, with only one wash step and enhanced sensitivity are also available. Some also have flexibility to perform either single or multi-target analysis on the same plate. (Abcam, eBioscience, ENZO, TGR Biosciences).

Cellular assays: Cellular assays are ideally suited for analysing a specific signalling event within a pathway in response to environmental cues. Assays are available to measure protein phosphorylation, acetylation, ubiquitination and histone methylations, providing novel high-throughput alternatives to more commonly used western blot analysis and ELISA-type assays. (Caliper, Corning, Life Technologies, PerkinElmer, TGR Biosciences).

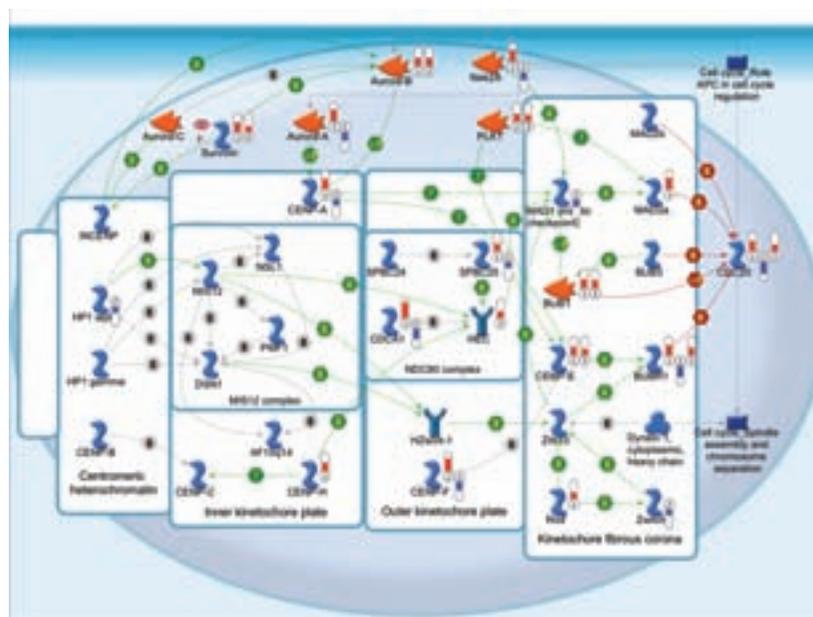**Reporter assays:** New reporter assays include those

Figure 28: The pathway map 'The Metaphase Checkpoint' from MetaCore collection (Thomson Reuters). The symbols depict proteins of different function. The arrows show protein interactions: green for activation; red for inhibition. 'Thermometer'-like histograms represent microarray gene expression experiments for lung cancer mapped on the pathway. Red histogram shows up-regulation of certain genes in cancer; blue histograms shown down-regulations

supporting specific signalling pathways (eg Wnt and NFAT), some enable a multi-pathway cellular profiling approach and others include a unique collection of experimentally-validated, fully characterised reporter assays that can be used to measure the effects of compounds on a variety of biological pathways. (ENZO, Life Technologies, Promega, Switchgear Genomics).

Multiplexed immunoassays: Bead-based multiplexed detection on Luminex® platforms are being increasingly utilised to analyse site-specific phosphorylation events and to support cell signalling assays for the simultaneous measurement of multiple phosphorylated proteins in cell culture and tissue samples. Antibody arrays are also being used to simultaneously measure relative changes in the phosphorylation of multiple receptor tyrosine kinases (RTKs) and cytoplasmic kinases involved in signal transduction. (Bio-Rad, EMD Millipore, R&D Systems).

Pathway profiling services: A range of contract research services designed to study key disease-related signal transduction pathways are now offered. Drug compounds can be rapidly profiled across many different signalling pathways to validate lead compounds, determine dose-response, indicate

Pathway Analysis

References
1 Pathway Analysis (Signal Transduction) Trends 2011 Report, published by HTStec Limited, Cambridge, UK, September 2011.
2 Doyon, JB et al (2011). Rapid and efficient clathrin-mediated endocytosis revealed in genome-edited mammalian cells. Nature Cell Biol.13(3):331-337.

whether signalling feedback or cross-talk is interfering with a compound's effectiveness, and pinpoint potential off-target effects. (Caliper, Corning, EMD Millipore, Life Technologies, Switchgear).

Informatics: Software and database applications are available that can quickly understand gene expression data, identify novel biological insights and generate testable hypotheses to drive the experiment-to-experiment cycle. Others integrate millions of literature-derived facts on protein interactions, pathways and biomarkers and use data mining interface with 'systems biology' tools for ontology enrichment, interactome mapping and network analysis. (Ingenuity, Elsevier, Thomson Reuters).

New imaging and analysis techniques: These simplify, expedite and increase the throughput of numerous pathway analyses including: receptor desensitisation, stem cell differentiation, inflammatory response, host signalling response to infectious agents, cancer biology and neurogenesis. Other approaches include creating immortalised cancer cell lines, having key signalling pathway and cytoskeletal genes tagged with fluorescent reporter proteins. Live cell imaging is then used to study protein localisation and subcellular translocation in response to various stimuli such as growth factors, cytokines and/or drug treatments. (Molecular Devices, Sigma Life Sciences).

RNA Signalling: Immuno-affinity methods of exploring RNA-protein and RNA-RNA interactions give researchers a biological approach to finding functionally related genes of disease pathways. (MBL Intl.)

Gene expression: New tools integrating real-time PCR into robust turn-key solutions for systems biology research facilitate pathway-focused profiling of gene and miRNA expression together with DNA mutation and methylation status. When coupled with best-in-class bioinformatics for gene content, these enable smaller labs to gain insight into gene networks. (Qiagen).

Other technologies: Other technologies are also being used to detect, characterise and quantify the level of proteins and nucleic acids involved in signalling pathways, eg digital PCR; SPR to measure protein interactions; antibody-based flow cytometry method to measure functional inhibition of the kinase targets; and imaging flow cytometers to analyse signal transduction in heterogeneous cell populations and correlating signalling events with phenotypes. (Bio-Rad, Caliper, EMD Millipore).

The above trends point to a wide diversity of tools that are now available to support signalling pathways research. The identification and characterisation of the components that make up the cellular signalling pathways will unquestionably remain a subject for in-depth investigations in the years to come.   DDW

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