Ion channels and stem cells

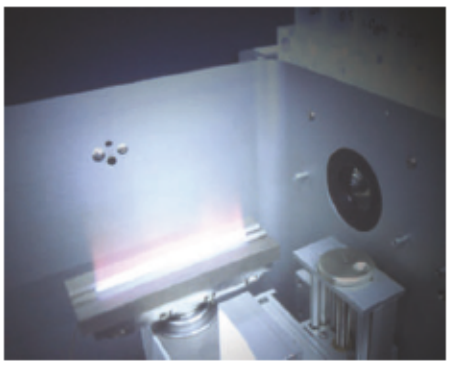
Ion channels, stem cells and cell signalling are the focus of intense interest in both cell biology and drug discovery. Pete Moore takes a look at what's on offer for the researcher.

Ion channels act as electrical gatekeepers in cell membranes, and are responsible for the generation and propagation of nerve impulses, muscle contraction, and many other biological processes. With more than 400 ion-channel genes identified in the human genome, interest in detecting and measuring their activity is burgeoning.

A high-throughput method of assessing the function of outward-rectifying potassium channels is to monitor the flow of tracer ions through them. In the case of potassium channels, rubidium ions (Rb^+) are used as a tracer because Rb^+ has similar characteristics to K^+ but is not present in biological systems and so there is no background noise. Trace amounts of Rb^+ (as low as 0.05 mg l^{-1}) can be detected using flame atomic absorption spectroscopy with the Ion Channel Reader (ICR) from Aurora Biomed of Vancouver, British Columbia. The ICR can be used to study voltage- and ligand-gated potassium channels as well as sodium channels and chloride channels.

Another way of studying ion-channel activity is to monitor changes in membrane potential. Invitrogen of Carlsbad, California, and PerkinElmer of Boston, Massachusetts, have

AURORA BIOMED



Aurora's Ion Channel Reader measures Rb^+ flow.

recently joined forces to offer a combination of Invitrogen's Voltage Sensor Probes ion-channel reagents and PerkinElmer's CellLux Fluorescence Cellular Screening Platform. This assay is based on fluorescence resonance excitation transfer (FRET); it uses a coumarin-phospholipid FRET donor that binds to the exterior of the cell membrane and a negatively charged FRET acceptor. In resting cells the two probes associate with the membrane exterior, resulting in efficient FRET and a red fluorescence signal. When a cell becomes depolarized as ions flow through channels, the FRET acceptor rapidly translocates to the other membrane face. Exciting the donor probe now generates a blue fluorescence signal.

Tracking channels

Ion-channel localization can affect cell function dramatically, and ChanTest of Cleveland, Ohio, offers antibody-based tests for detecting intracellular ion-channel trafficking. "In cystic fibrosis, 50% of families have a defect that prevents the CFTR channel protein being transported to the cell surface, and for the hereditary form of the hERG disease, about half of the mutations in the hERG channel protein affect trafficking," says ChanTest's chief executive officer Arthur 'Buzz' Brown. Blocking the function of the hERG potassium ion channel in cardiac muscle may be a major adverse drug effect as it can cause arrhythmia and sudden cardiac death, and all new drugs must be tested for whether they block this channel. In ChanTest's HERG-Lite assay, human embryonic kidney (HEK) cells express a version of the hERG channel carrying a hemagglutinin epitope. Protein turnover replenishes hERG channels about every 12 hours, so the cells are incubated overnight with

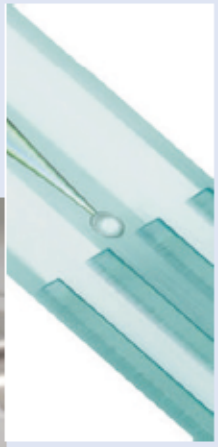
MAXIMIZING RETURN

CELLECTRICON

Although most patch-clamp technologies seek to maximize the number of cells rushed through the system, Owe Orwar and his colleagues at Cellectricon, a start-up company based in Gothenburg, Sweden, have developed a platform that maximizes the information gained from each cell. The result is a powerful tool for secondary screening in drug discovery. Their Dynaflow technology uses conventional glass pipette patch clamping, in combination with a novel microfluidic device for controlled delivery of drug solutions.

Solutions of drugs or drug combinations are placed in up to 48 wells, each of which is connected to a measurement chamber by micrometre-diameter channels. These solutions can be directed through the chamber with high precision. "Dynaflow uses the unique properties of fluids when they are running at very low Reynolds numbers. When the fluids come out from a tiny channel in the open volume they behave as if they are still in channels — they do not mix," says Orwar.

Cellectricon's Dynaflow patch-clamp system combines microfluidics and patch-clamping technology.



With no turbulence, diffusion would be the only chance of mixing between solution batches, but the timescales used are too short for that to occur. Consequently, Dynaflow can provide step changes in drugs or drug concentrations, with a change every 30 milliseconds if desired. "It is the most precise technology in the world to titrate receptors," claims Orwar. "You can see it as a microfluidic device that generates a barcode of chemicals, and the cell effectively reading the barcode," he adds.

The ability to squeeze so much data out of a single cell enables some users to claim a ten-fold increase in productivity. By using carefully considered combinations of drugs in each well, cells can be taken through physiologically relevant conditions that relate to many different disease states. "In effect, it gives you the option of passing a chemical waveform over the cell while constantly recording from it," says Orwar. P.M.

the test compounds. The next day an antibody for the epitope is added along with a second antibody that produces a chemiluminescent signal. "If you don't permeabilize the membrane you can count the number of channels at the cell surface — it's simple and fast," says Brown. ChanTest's FAST & Lite service runs the antibody-based test alongside automated patch clamping to assess channel function. Assays for other channels are being developed and ChanTest has been awarded a small-business innovation grant from the National Institutes of Health to automate its system.

Patch clamping goes automated

Although indicator-based methods are fast and inexpensive, the gold-standard for assessing ion channels is the Nobel prize-winning technique of patch clamping developed by Erwin Neher and Bert Sakmann in the 1970s. The conventional manual method involves a glass micropipette filled with an ionic solution that electrically connects a silver–silver chloride electrode wire to a small patch of cell membrane. A vital part of the procedure is to get an electrical seal of at least 1 gigaohm between the pipette tip and the membrane; without this seal the tiny currents that pass through the channels in the membrane patch cannot be measured. The drawback is that the technique requires considerable expertise, hours are spent poring over a microscope, and recordings can only be taken from one cell at a time. But over the past few years automation has entered this green-fingered science.

A major player in the automated patch-clamp market is Molecular Devices of Sunnyvale, California, which merged last year with imaging specialists Axon Instruments. Molecular Devices has two high-throughput automated patch-clamping systems that can collect between 100 and 2,000 patch-clamping data points a day, depending on configuration.

IonWorks Quattro from Molecular Devices.

MOLECULAR DEVICES

Both instruments work by sucking cells down against 1–2 μm diameter pores in the base of multi-well plates. The PatchXpress 7000A uses 16-well, glass SealChip plates made by Aviva Biosciences of San Diego, California. The machine places cells in each well and suction holds one cell that falls on the pore in place with sufficient strength to create an electrical seal of 1 gigaohm. The machine uses suction to disrupt the cell membrane to access the interior of the cell, and currents are measured across the entire cell surface. "You are, in effect, reversing traditional patch clamping by having the ground electrode measuring from the inside of the cell rather than from the outside," says Steve Davenport, vice-president of Europe for Molecular Devices. Each well is controlled and monitored individually and cells can be sealed for 30 minutes or more — during which time test compounds can be added to and flushed from the well. A single run takes around 45 minutes. The PatchXpress platform works well for both voltage-gated and ligand-gated ion channels and yields high-quality data comparable to the conventional manual patch-clamp method.

IonWorks Quattro from the same company uses a 384-well Patch Plate, but wells share electronics. "This makes sense for a screening instrument where you need the highest throughput possible without compromising the pharmacology," explains Davenport. The system uses a new technology developed by Molecular Devices called Population Patch Clamp (PPC). PPC uses 64 holes versus a single hole in each well of the Patch Plate. This enables the signal from up to 64 cells in each well to be averaged. "The advantage of PPC over conventional single-hole planar patch-clamp is the reduction in biological variability and substantial increase in the success rate of obtaining a data point from each measurement," says Davenport. Using IonWorks, scientists can measure up to 2,000 data points per day.

This speed doesn't come cheap. Both

STEM-CELL OPTIONS

It's easy to focus on the kit and forget the really important part of the system — the cell. Cells of most interest with respect to ion channels include neurons and heart cells, which cannot be grown for long in culture and do not divide.

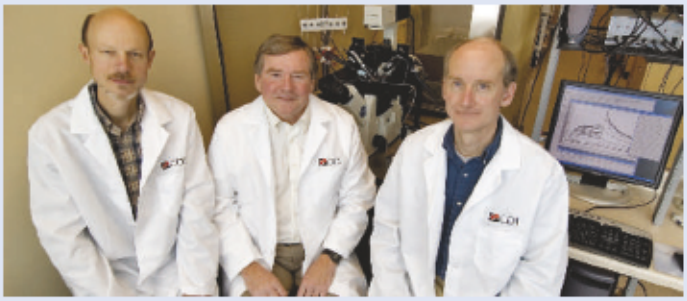
Many of the cell lines used in ion-channel work are, therefore, stem cells and cell lines engineered to express specific channels. These include human embryonic kidney (HEK293) and Chinese hamster ovary (CHO) lines. bSys of Basel, Switzerland, offers a wide range of screening techniques, but chief executive officer Daniel Konrad believes that one of the company's chief advantages is their skill in selecting and fine-tuning cells.

"Each clone of cells is subtly different, and only trialling with many different sources can show which expression system is ideal," he says. bSys also works hard to find the right suspension protocol. This can make the difference between cells that generate 200 picoamp currents and those that can generate 500–1,000 picoamps and can be used in robotic screening systems, says Konrad.

A new company moving into the designer-cell niche is Cellular Dynamics International (CDI) of Madison, Wisconsin, founded by noted human embryonic stem-cell researchers James Thomson, Craig January and Timothy Kamp of the University of Wisconsin. CDI will initially focus on developing HEK cell and cardiomyocyte-based screening services to the pharmaceutical and biotechnology industries, and plans to have a drug-screening service running by the first quarter of 2006.

On the other side of the Atlantic, in Edinburgh, UK, the European arm of Stem Cell Sciences, founded by Peter Mountford in Melbourne, Australia, is developing neural stem (NS) cell lines from the Universities of Edinburgh and Milan. These cells are thought to be phenotypically similar to the NS cells found *in vivo*. Derived from human and animal embryonic stem (ES) cells and from fetal and adult brain tissue, NS cells have great potential in biomedical research because of their homogeneity, their ability to self-renew indefinitely, and their relative ease of manipulation. Stem Cell Sciences is establishing a service for generating specifically mutated NS cells from engineered ES cells and transgenic animals. NS cells are attractive candidates for *in vitro* drug screening and may also be useful for cellular therapy for conditions such as Parkinson's disease and epilepsy.

R&D Systems of Minneapolis, Minnesota offer ready-to-use primary cortical stem cells derived from rat embryos and the kits to grow them. The cells are validated for differentiation into astrocytes, neurons and oligodendrocytes. P.M.



B. FRITZ

Cellular Dynamics International: James Thomson (right), Timothy Kamp (left) and Craig January.

machines cost up to US$400,000. And although the IonWorks platform works well for voltage-gated channels, where you can adjust the voltage at the same time as recording, it will not work for fast ligand-gated channels, whose currents often last a millisecond or less, as the machine cannot add test compounds and record simultaneously.

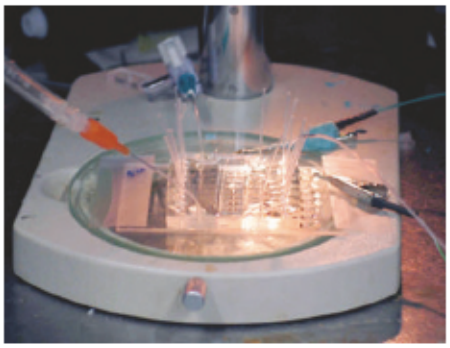
Contenders aiming to overcome the ligand-gated channel barrier in automated patch clamping also include Sophion Bioscience of Ballerup, Denmark, which uses a microfluidics approach. Its QPatch 16 operates 16 independent patch-clamp sites, each comprising a flat silicon chip with recording electrodes, a patch-clamp hole, pipetting wells and integrated microfluidic glass flow channels for applying solutions. "QPatch 16 also provides a cell preparation facility in which the cells are suspended in culture medium until right before the experiment. This ensures that cells are kept viable and healthy, and enables unattended operation for at least 4 hours," says Niels Willumsen, a senior executive at Sophion. The integrated microfluidic flow channels of the QPlate allow sequential application of multiple compounds at very low volumes (around 5 μl) from four to eight pipette tips, and ensure the fast solution exchange (about 50 ms) required to study ligand-gated ion channels. The modular design can be upgraded to a 48-channel system and the machine can give 250–1,200 data points per working day.

On a smaller scale, Fred Sigworth and Kathryn Klemic at Yale University, New Haven, Connecticut, have developed a planar patch clamp that can be built in the lab. "In the future, instead of buying an expensive chip, a lab might have a little device that can make an electrode, or an array of little electrodes, by moulding them out of silicon rubber," says Sigworth. A thin layer of polydimethylsiloxane (PDMS) resin is poured on to a plate containing a 2-μm diameter hole. Before the PDMS cures, air is blown through the hole, creating a 1-μm hole in the rubber sheet. After peeling the sheet off the plate, exposure of the surface to plasma oxidation creates a 100-μm thick glassy surface layer of SiO_2. "On the one hand you have a hydrophobic silicone rubber base, then you create this thin layer of glass that the cell rests on — to a cell it looks a lot like a conventional glass electrode," says Klemic.

In expert hands, the best systems for patch clamping can currently detect a pulse of about 150 elementary charges: equivalent to a flow of 150 sodium ions. "The grand challenge would be to resolve single elementary charges. Then you could watch a lot of really interesting processes such as the turnover of ions in pumps," says Sigworth. He is unsure whether this single-ion resolution will ever be possible, but thinks that it may be possible to mould the PDMS sufficiently carefully to reduce the capacitance in the system and substantially increase the resolution.

NANION

Nanion's Port-a-Patch makes patch clamping easy for the novice.



F. SIGWORTH & K. KLEMIC

Do-it-yourself: the PDMS microfluidic patch-clamp system in use.

Sigworth is also intrigued by the Port-a-Patch system developed by Nanion Technologies, a spin-off from the Centre for Nanoscience at the University of Munich in Germany. The beauty of Port-a-Patch is its ease of use. "It's basically a bench-top patch clamp. You pipette in the cells, close the lid and make the recording," he says. Nanion claims that this turn-key solution only takes half an hour to set up. "We run one-day training courses, and the system is easily used by people who have no experience in electrophysiology," says Nanion's

BANKING ON STEM CELLS

Human stem cells are valuable commodities: as well as their medical potential, their pristine naivety makes them attractive as gold-standard cell lines for research. Stem-cell banks, where owners deposit their precious products and would-be investigators apply for loans, are now being developed.

The most advanced is the UK Stem Cell Bank, based at the National Institute for Biological Standards and Controls in Potters Bar, near London. Initiated in September 2002, and funded by the Medical Research Council and the Biotechnology and Biological Sciences Research Council since January 2003, it has the aim of providing a repository for all types of human stem-cell lines.

"As of October 2005, we have 24 stem-cell lines approved for accession into the bank," says director Glyn Stacey, but none is yet ready for sending out to end-users. That probably won't be until early 2006. "The process is complex. It is not like growing an ordinary cell line where you could create and quality control a bank within a few months of receiving the cells," says Stacey. One time-consuming step is the creation of agreements for depositers and recipients, with each cell type presenting different problems and opportunities. Exploitation will be controlled by the depositer who retains ownership of the cells.

Legal issues aside, stem cells are challenging to grow. The main problem is scaling up to provide hundreds of ampoules of cells at identical passage levels and stages of differentiation. "It could take an entire day for a highly skilled person to dissect and recover cells from just one line," says Stacey. And cultures have to be characterized and checked for contamination before release.

All lines currently in the bank are human embryonic stem (ES) cells. "We have had some contact with people who think they have adult stem cell lines, but they are being careful about characterization," says Stacey.

MRC

Glyn Stacey: stem cells are challenging to grow.

A few ampoules of each cell line have been frozen as back-up, whereas the master bank contains 20 or 30 ampoules. The distribution stock may eventually contain around a hundred ampoules of each line. Stacey hopes that early in 2006 the bank's website will start tracking progress of the cell lines that will be available to researchers.

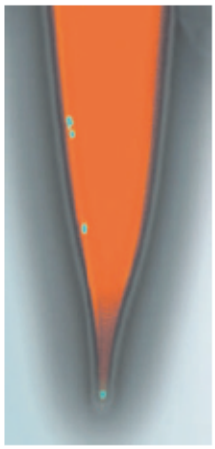
A few other initiatives are taking shape. The US National Stem Cell Bank will be located at the WiCell Research Institute, in Madison, Wisconsin, with a $16.1 million, four-year National Institutes of Health grant. It will acquire, store, characterize and distribute human embryonic stem-cell lines, but will be limited to those approved for federal funding. After a year of legal wrangling, a stem-cell bank is taking shape at the University of Granada in Spain, and others are being considered in Australia and South Korea. P.M.

chief executive officer Niels Fertig. It is the only automated device that addresses the need for low throughput with high accuracy, Fertig claims.

The Port-a-Patch system uses planar borosilicate glass chips (100-μm thick) in which a conical pore of 1-μm diameter is micromachined. The pore has the three-dimensional geometry of an inverted pipette tip and cells are simply positioned via suction. It creates a strong electrical seal with the cell and is ideal for whole-cell patch clamping. Single-channel recordings can be performed in a cell-attached configuration. A software-controlled eight-channel microfluidics add-on can deliver sufficiently rapid changeover of solutions to allow the study of ligand-gated channels. A robotic version of the system that will run 16 patches at a time is in prospect.

The Flyscreen, an alternative approach for moderate-throughput applications from flyion of Tübingen, Germany, can perform 100 to 500 independent whole-cell screens per day. The instrument uses glass micropipettes, into which cells are loaded. As the cells settle, a single one falls towards the tip and wedges near the opening. Carefully controlled suction draws the cell into a tight fit and further trains of pressure pulses disrupt the membrane, leaving a patch of cell membrane spanning the pipette's lumen. A plastic jacket moulded around the pipette enables robotic handling. The machine holds up to six pipettes and each channel runs independently, so pipettes can be discarded as soon as the cell fails.

"Glass blowing enables us to be flexible in the shape and geometry of the tips," says inventor Albrecht Lepple-Wienhues, founder and chief executive officer of flyion. This allows tailoring to suit different types of cells, and the new Flip-the-tip Large tips, which have a bowl at the base, enable the machine to monitor ligand-gated channels. "The bowl at the base gives us enough space to intro-duce a 130-μm diameter quartz needle," says Lepple-Wienhues. In flyion's standard tips, solution exchange takes about 60 seconds, but the new tips allow solutions to be puffed directly on to the cell through the quartz needle and give exchange rates of less than 50 milliseconds, while continuous recording is being carried out from each cell.



FLYION

flyion recording tip with a cell held in place at the end.

Patch-clamp economics

A report published in September by the Cambridge-based market-research consultancy HTStec makes interesting reading for those involved in the ion-channel industry. According to HTStec, the pharmaceutical and biotech market will spend around $32 million in 2005 on automated patch-clamping machines. "We predict that sales will peak in 2006 at around 200 units a year," says HTStec director John Comley. In addition to this, the report estimates that for automated patch-clamping, labs spend around $10 per data point for safety assessment and $3.00 per data point in primary screening. Companies claim they would be more comfortable paying around $6 and $0.60 respectively. HTStec's survey of 66 companies and universities indicated that manual patch clamping was still the preferred option in assay development and safety assays such as hERG compliance, but automated patch clamping was the method of choice for secondary screening, lead optimization and early non-compliant hERG liability testing.

The highest possible throughput of some 3,000 data points a day is still far short of the 20,000 data points that respondents said they would like to get from a machine. Many were looking forward to machines that measure ligand-gated channels much more cheaply. As these account for 29% of all ion channels studied, this is a potentially big market.

With genomics and proteomics creating a resurgence in cellular and systems research, there is every reason to believe that ion-channel research will become even more important in the coming decade. ■

Pete Moore is a science writer based near Bristol, UK.

Sigworth Laboratory
▸ info.med.yale.edu/cmphysiol/sigworth/

HTStec
▸ www.htstec.com

SIGNALS OF DISEASE

Protein kinases are linked to numerous disease states, including cancer, arthritis, diabetes, cardiovascular diseases and neurological disorders. Gleevec from Novartis was the first compound active against a kinase (the Abl kinase) to be approved as a treatment — for certain gastrointestinal tumours and chronic myeloid leukaemia.

The market for kinases is large. "More than 25% of new drugs being developed today are based on kinase technology," says Jeff Linton, president of Upstate of Charlottesville, Virginia, which offers one of the largest collections of kinases. A flagship of Upstate's operation is its KinaseProfiler service, run from Dundee in Scotland. This provides quantitative characterization of compounds against an ever-expanding panel of human protein kinases in a direct radiometric assay. The panel currently contains around 230 kinases, almost 50% of the total number of human kinases in the genome. A new focus for Upstate is the addition of naturally occurring mutant kinases as they are identified.

R&D SYSTEMS

R&D Systems' Phospho-MAPK array tracks phosphorylated kinases.

Attention is also focusing on the newly emerging Gleevec-resistant mutants of Abl, and mutant forms of other kinases including the epithelial growth factor (EGF) receptor, as these mutations can alter an inhibitor's efficacy. One of these mutations involves a single 'gatekeeper' amino acid. Mutations in this amino acid can prevent therapeutic compounds from binding effectively without affecting the enzyme's activity. "The search is on for successful inhibitors that are not sensitive to changes at the gatekeeper site," says Steve Davies, director of Upstate's drug discovery segment.

Upstate is helping this search by adding eight different mutant kinases to their portfolio, including ones for Kit, EGFR, Abl, Flt3 and p38/SAPK2a — and, says Davies, there are more in the pipeline.

A highly specific set of anti-kinase antibodies makes up R&D Systems' Proteome Profiler Phospho-MAPK Array. This allows analysis of the phosphorylation status of 19 key signalling proteins, including members of all three major families of mitogen-activated protein kinases — the extracellular signal-regulated kinases, c-Jun N-terminal kinases, and the p38 kinases. These enzymes play essential roles in numerous signalling pathways that underlie cell function and disease.

Signalling pathway analysis products from Beckman Coulter of Fullerton, California, are also devoted to looking at intracellular activated (phosphorylated) kinases. One strength is that these reagents can be used on many different types of specimen including whole blood, and can resolve activated and inactivated kinases in whole blood cells, according to Michel Herbert, marketing manager for Beckman Coulter. P.M.