Divide and conquer

A key element of performing good cell-biology experiments is starting with exactly the right cells. Michael Eisenstein takes a look at the technologies that can make this possible.

What do you get when you cross an ink-jet printer with a Coulter counter? It's not a riddle; scientists at Los Alamos National Laboratory in New Mexico asked the question 40 years ago, and the answer turned out to be the cell sorter.

Los Alamos researcher Mack Fulwyler created a prototype in 1965, which used vibration to generate tiny droplets from a jet of solution containing red blood cells; the individual cells in each droplet could then be subjected to rapid volumetric analysis and sorting. Fulwyler's prototype came to the attention of Stanford University researcher Leonard Herzenberg. "I was working on immunofluorescence in immunology and genetics," he says, "and had realized that there was a need for the means to sort cells according to the molecules they display on their surface."

Herzenberg and his colleagues adapted Fulwyler's design to produce an instrument that could sort cells depending on the presence or absence of molecules identified by fluorescent labels — the first example of fluorescence-activated cell sorting (FACS). Today, FACS has not only aged gracefully but is arguably in its prime, embraced by nearly everybody looking to pluck specific cells out of complex mixtures. Herzenberg's patent was licensed by Becton Dickinson (BD), which developed the first commercial instrument and currently holds the trademark for the term 'FACS'. Early FACS fans include Dutch researcher Gerrit Van den Engh, whose refinements enhanced sorting rates. "I designed a different, digital parallel post-processing scheme," says Van den Engh. "By digitizing the signal as quickly as possible, we could take the cells in parallel and we could put an error-tracking code on the information, so that we could check whether events were being dealt with properly."

L. HERZENBERG

Leonard Herzenberg devised early cell sorters.

Cytomation, later acquired by Dako in Glostrup, Denmark, made Van den Engh's patent the foundation for MoFlo, the first high-speed commercial sorter. MoFlo pushes the speed envelope, sorting up to 70,000 objects per second — although researchers often use lower rates to optimize recovery and sort accuracy. Dako has also ensured that new expansions and components are suitable for use both with current and older systems. "Our modern upgrades are reverse-compatible, even with MoFlo machines from eight or nine years ago," says Cytomation's founder, George Malachowski.

Meanwhile BD Biosciences, a segment of BD in San Jose, California, recently introduced its most advanced cell sorter to date. The BD FACSAria benefits from its small size, being one of the few bench-top systems on the market, and from an alternative approach to pre-sorting analysis. Most sorters use the 'jet-in-air'

THE GENTLE TOUCH

Sorting individual cells is not a problem, but what if a researcher wants to sort clusters of cells, or even whole embryos?

Union Biometrica of Holliston, Massachusetts, offers a potential solution in the shape of its COPAS family of instruments. These, it says, can work with objects ranging from pancreatic islets up to zebrafish hatchlings. The principle is similar to fluorescence-activated cell sorting (FACS) — objects in solution pass through a specially designed flow-cell, where they are optically profiled according to as many as five different sorting parameters (which can include three fluorescent wavelengths), and specimens of interest are diverted and collected.

But some design adjustments were necessary to protect the integrity of the multicellular objects being sorted. For one thing, rather than using charge-based sorting of droplets, COPAS uses rapid puffs of air to gently divert 'slices' from the flow stream that contain objects of interest.

COPAS also uses lower flow-rates than FACS, sorting between 100 and 300 objects per second. "We sacrifice speed for gentle flow, which results in increased viability and less destruction of what we're analysing," says Rock Pulak, Union Biometrica's director of life sciences. "It's still much faster than trying to analyse these same numbers of cell clusters by microscope."

UNION BIOMETRICA

Union Biometrica's BioSorter COPAS can sort clusters of cells.

In addition, the lower speeds mean that it is possible to do limited analysis of fluorescence localization within the objects being sorted. Pulak speculates that future instruments may even be able to perform actual imaging during the sorting process.

Sometimes even single cells prefer a lighter touch, and an ongoing collaboration between the company and the Joslin Diabetes Center in Boston has shown that COPAS is also useful for sorting adipocytes and hepatocytes.

"These are larger cells that are very delicate and subject to sensitivities with respect to shear force," says Pulak. "We are finding that our technology is appropriate for these kinds of applications." M.E.

approach, in which optical analysis is performed on a rapidly moving stream of fluid, but the BD FACSAria instead uses a sorting flow-cell. "This gives you the much higher sensitivity that you need, but maintains extremely efficient sorting," says marketing director Tony Ward. "And you can sort cells that have lower levels of antigen expression than you might be able to see using a jet-in-air approach."

Several alternative systems are available. Beckman Coulter of Fullerton, California, another early entrant into the field, offers its EPICS ALTRA, an established platform for cell sorting. In 2000, Van den Engh launched a new company, Cytopeia, based in Seattle, Washington, whose inFlux Cell Sorter is based on ideas from his academic work. "It's an open system, so you can have access to all the modules and can configure it freely for whatever experiment you want to do," he says. "We're not competing with the other manufacturers for well-established applications; we work with the 10–20% of researchers who have applications that are not done as well on the other machines." And for researchers working with larger objects, Union Biometrica of Holliston, Massachusetts, offers a FACS-like platform for sorting embryos and multicellular clusters (see 'The gentle touch', page 1179).

Most observers agree that cell sorting has probably reached its speed limit, and some scientists are now looking to expand the breadth of flow cytometric analysis and sorting. Mario Roederer of the US National Institutes of Health (NIH) in Bethesda, Maryland, has been a leader in this regard, combining fluorescent dyes and quantum dots to perform experiments involving simultaneous analysis of up to 17 different intracellular and cell-surface markers. Many cell biologists have yet to explore these outer limits, but current commercial sorters can typically accommodate optics for analysing a dozen or more fluorescent parameters. As an immunologist examining very specific cell subtypes, Roederer finds this flexibility invaluable: "We're routinely doing 12- or 15-colour flow-cytometry to try to look for important subsets or functions. In the end, I'm hoping we'll be able to reduce it to a 4- or 5-colour assay with the correct combination of markers."

BD BIOSCIENCES

The BD FACSAria uses a sorting flow-cell.

With all the power that these cell-sorting systems offer, there are still problems to be resolved. "Software is the issue that requires the most effort," says Roederer. "We need tools that can automate the discovery or the analysis of subsets of cells that are present in complex data sets." Ward agrees: "The faster you count particles, the more data get generated and the resulting high degree of data complexity and intersections mean that current approaches to software can be limiting." Both Roederer and Herzenberg have worked to address this, developing two commercially available software packages, FlowJo and FacsXpert, intended to improve the quality of cell-sorting analysis.

Another big factor for many is cost: power and efficiency don't come cheap, and access to high-end machines may be restricted to limited slots in a shared core facility. "I'd like to see cheaper machines that give you five, six or seven colours but that are much less expensive than the mammoth machines," says Herzenberg.

Nevertheless, these instruments receive strong acclaim from users. "I don't want to say it's a way of life," says Roederer, "but it is a way of biology."

Working in bulk

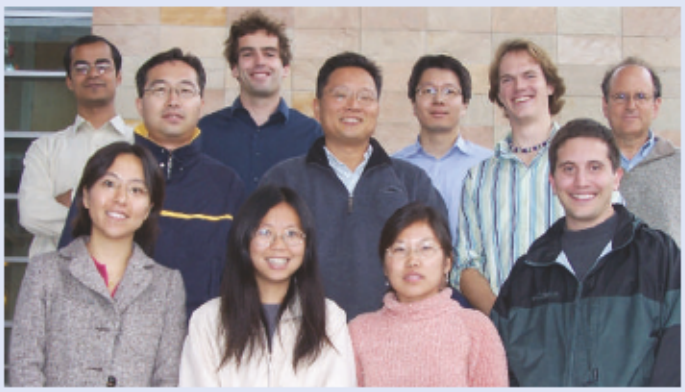
Sometimes, however, all a scientist needs is a way to separate two groups of cells quickly. "FACS can do pretty much everything, but it's expensive," says Steven Woodside, a scientist with StemCell Technologies of Vancouver, British Columbia. "If you want to do more bulk separations, then immunomagnetic separation is a really good option."

The principle is simple. Cells are incubated with paramagnetic beads tagged with antibodies, after which a magnet or array of magnets can be used to either purify cells of interest or remove unwanted cells. Dynal Biotech's Dynabeads — currently available through Invitrogen in Carlsbad, California — were among the first

PLAYING THE FIELD

Researchers have used electrical fields to manipulate nucleic acids and proteins for more than 50 years, but similar systems have only recently begun to emerge for working with whole cells. Dielectrophoresis is nevertheless quickly gaining appeal as a basis for microfluidic cell-sorting.

Hyongsok (Tom) Soh and Patrick Daugherty at the University of California, Santa Barbara, recently demonstrated the feasibility of dielectrophoresis-activated cell sorting, or 'DACS', with a microfluidic chip that uses dielectrophoretic forces to steer bacteria tagged with polystyrene beads into a collection channel (X. Hu *et al. Proc. Natl Acad. Sci. USA* 102, 15757–15761; 2005). Initial experiments showed that one round of DACS could achieve more than 200-fold enrichment of a rare subpopulation of cells at rates of 10,000 bacterial cells per second. They initially tested a single-stage, single-channel device, but Soh believes DACS is ideal for parallel operations. "It is relatively straightforward to design cascaded, sequential sorting stages that operate in parallel," he says. "This allows high purity and cell recovery without sacrificing throughput."



H. SOH

Tom Soh (middle row, centre) and his team have put together prototype microfluidic chips for dielectrophoretic cell-sorting.

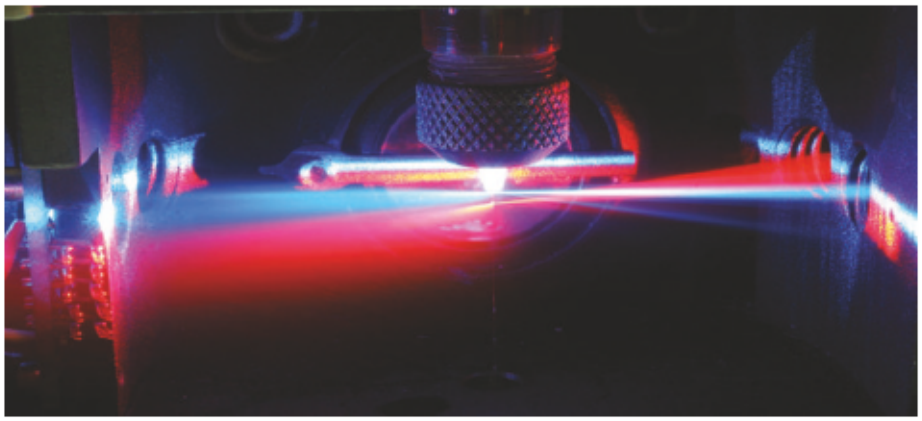
Soh is quick to point out that DACS is in no position to usurp fluorescence-activated cell sorting, because of the binary nature of its sorting mechanism; but it shows great promise for high-throughput screening, he says. "We just completed screening the first molecular library and performed epitope mapping with DACS," Soh explains, "and we've shown that it can be faster, cheaper and simpler than commercial assays."

Evotec Technologies in Hamburg, Germany, is also taking advantage of dielectrophoresis for its Cytocon 400 system. The key to this is the CellProcessor microfluidic chip, which contains a three-dimensional array of electrodes that allow users to design and control electrical-field configurations for cell manipulation.

"We developed the CellProcessor platforms for precise and fully automated sorting in a microfluidic environment," says Gabriele Gradl, Evotec's vice-president of cell handling and analysis. "The underlying technology makes cell analysis and isolation reproducible and predictable down to the single-cell level." The resulting platform allows for the delicate manipulation of small numbers of cells, in which the gentle handling provided by combining dielectrophoresis with hydrodynamic flow can be useful.

M.E.

DAKO



Inside Dako's MoFlo, which can sort up to 70,000 objects per second.

such commercial products, and remain a popular option. Miltenyi Biotec, based in Bergisch Gladbach, Germany, also offers reagents for performing column-based 'magnetic-assisted cell sorting' (MACS) using its MACS Separator. This is available in an automated version to simplify the purification process.

StemCell's offerings for magnetic separation include the EasySep system, which uses a specially designed high-gradient magnet to separate nanoparticle-labelled cells. These paramagnetic particles are particularly small, to an extent where they will not interfere with flow cytometry performed on purified cells. StemCell also offers an automated version, the RoboSep, which gives users a variety of options for configuring separation protocols.

Magnetic separation's usefulness is limited by the inherent constraints on the number of sort parameters and its reliance on cell-surface antigens for sorting. Nonetheless, it excels at applications in which bulk separations need to be performed quickly, or as a prelude to more extensive cell-sorting procedures. "In a lot of cases for immunology research, the level of purity that you get with magnetic separation is more than adequate," says Woodside. "Basically, people want to do the fewest steps possible and get the purest cells back — that's what's driving this technology."

Bridging the gap

With all the interest in optimizing the efficiency and cost of cell sorting, it is understandable that James Leary, head of the molecular-cytometry facility at Purdue University in West Lafayette, Indiana, is disappointed at the chilly reception that microfluidic platforms tend to receive in the community. "Flow cytometry has had microfluidics at its core for 40 years," he says. "But it's interesting, because the flow-cytometry groups and the microfluidics groups don't talk to each other very much. If they did, progress would be a whole lot faster."

Leary, a cell-sorting pioneer, is doing his part to bridge that gap through collaborations with colleagues such as Rashid Bashir, from his university's Birck Nanotechnology Center. The two see great advantages at the microscale, including portability, disposability and improved biosafety for handling pathogenic samples. They are exploring next-generation sorting technologies such as dielectrophoresis, in which a nonuniform electrical field is used to separate charge-neutral objects based on their size or chemical properties (see 'Playing the field', page 1181). "The dielectrophoretic approach is attractive because it is electrical, it is integrated and you don't have to have lots of mechanical valves," explains Bashir. "Moving the cells, instead of the fluid, makes more sense."

The use of laser light for cell manipulation is well-established (see 'The guiding light', below), and lasers are now being exploited for cell sorting. Kishan Dholakia, leader of the optical-trapping group at the University of St Andrews, UK, recently developed a system in which two- or three-dimensional patterns are generated by an optical-tweezers laser to create a 'passive' cell-sorting matrix. "We put cell samples on to an optical corrugation or

THE GUIDING LIGHT

Never mind Star Wars, lasers are good for more than burning and cutting, and in fact can be surprisingly gentle. Take 'optical tweezers', for example — a laser focused on a microscopic object creates an optical force trap that enables the precise manipulation of that object within a three-dimensional space.

Since their development in 1986, optical tweezers have generated a veritable bounty of valuable information, including insights into the physical properties of DNA molecules and the fundamental mechanisms of various enzymes and molecular motors.

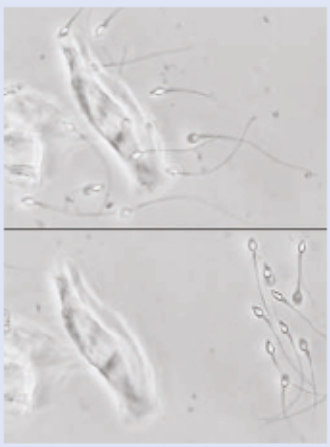
They have also shown considerable promise for cell manipulation, although biologists have yet to fully explore their potential. "We have to educate people and make them comfortable with optical technology," says Kishan Dholakia, who heads the optical-trapping group at the University of St Andrews, UK.

Many current users build their systems from scratch — a daunting and expensive prospect for the optics novice. To remedy this, Dholakia's team has lent its expertise to develop an entry-level, single-beam optical-tweezers workstation — the E3100 — for biologists looking to get their feet wet. The E3100 is available from Elliot Scientific in Harpenden, UK.

Cell Robotics of Albuquerque, New Mexico, also offers an off-the-shelf system: LaserTweezers, an adjustable single-trap system that is designed for integration with a standard inverted microscope, and which can also be incorporated as a module of a larger workstation to allow computerized control and full automation.

Single-beam traps enable impressive experiments, but the future clearly lies in higher-throughput platforms. "The technology has always been associated with one to ten particles," says Dholakia. "I would like to see thousands of particles being ordered and sorted in a really rapid fashion."

Enter Arryx of Chicago, Illinois; its BioRyx 200 system uses holographic technology to expand the number of tweezers simultaneously available to users. "It can generate up to 200 traps in a three-dimensional working volume, each of which is independently movable in real-time," says chief technology officer Dan Mueth. "You can pull on cells and sense or measure how they stretch, grab cells and move them around, probe the adhesion of cells, position cells for investigation, or isolate cells."



ARRYX

Caught: sperm cells isolated from epithelial cells using the BioRyx 200.

The BioRyx 200 also offers a software interface that enables real-time manipulation or automation of the traps. But multiple traps are not the only benefit of holographic optical trapping. "You can select from a variety of trap shapes with different properties," says Mueth. "There are plenty of other advantages that are more subtle but can have an impact, and have to do with optimizing performance and the ability to work with particular samples." M.E.

L. LIOTTA

At the cutting edge: Lance Liotta pioneered laser-capture microdissection.

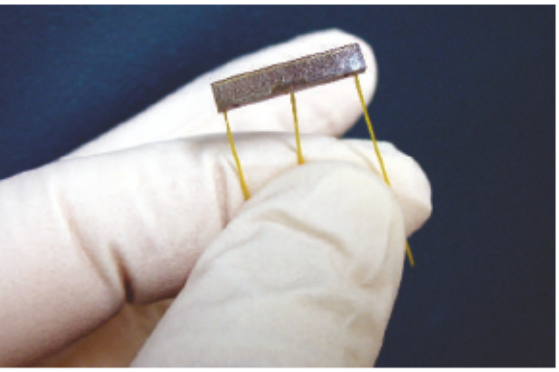
landscape," he says, "and this light pattern acts rather like an optical sieve." This proved effective for sorting lymphocytes from erythrocytes by size and shape, although Dholakia is still exploring how this might be used to sort cells with subtler internal differences.

Stanford University's Stephen Quake tested a more active sorting approach for his μFACS chip, integrating optical detection with electro-osmotic flow manipulation; when cells of interest are identified at a detection 'window', fluid flow at an adjacent T-channel is switched to divert the cell for collection. "What we have been using these cell sorters for is not as independent stand-alone things, but as an integrated component of a more complicated microfluidics system," explains Quake. His team has also developed micromechanical valve-based chips that function as part of a larger platform for single-cell genetic analysis.

Micromechanical valves are also the foundation of a chip developed by Innovative Micro Technology (IMT), a manufacturer of microelectromechanical systems (MEMS) based in Santa Barbara, California. IMT's rare-cell purification system (RCPS) is designed to purify stem cells from patient samples for transplantation, and chief executive John Foster suggests that its small size and disposability could make it ideal for clinical settings. RCPS chips feature 32 parallel channels that use tiny valves, optics and electromagnetic actuation to divert cells for rapid collection following detection of appropriate fluorescent markers; initial results have been promising. "We've shown that the human cells that we're sorting survive and reproduce, and we've got the speed, sterility, ease of use and disposability required for human-cell therapies," says Foster.

Microfluidics remains a hard sell for many, a niche dominated mainly by 'do-it-yourselfers', who design and build chips from scratch or with the help of companies specializing in biological microfluidics. Among other reasons, the perceived speed sacrifice of going micro remains a deterrent. But Bashir cautions that "the emphasis on speed is overplayed". Leary agrees. "We can do multiple channels and we can do sorting and re-sorting in a continuous-flow device instead of a droplet-based device — microfluidics can provide many other features," he says. In the end, the biggest problem may just be getting people to see an old problem in a new way. "It's hard to convince people, when there are already FACS machines that work very well, that they need another FACS," concludes Quake. "But people will start to use microfluidic FACS for all kinds of creative things if it's out there as a low-cost, personal alternative."

IMT



IMT's RCPS chip uses optical analysis and MEMS to isolate stem cells from patient samples.

Laser precision

The preceding systems offer a wealth of options for working with cells in suspension, but many scientists face the need to work with especially 'fresh' cells. "The process of disaggregating and sorting cells, and treating them over a time period will profoundly change the expression profile or protein-signalling pathways," explains Lance Liotta, co-director of the Center for Applied Proteomics and Molecular Medicine at George Mason University in Manassas, Virginia. In the mid-1990s, while working as a lab chief at the NIH, Liotta's frustration with mechanical methods for microscopic tissue dissection led his team to develop laser capture microdissection (LCM), a quick and precise system for excising cells from fixed tissue samples or even live adherent cultures.

The patents from this work were developed by Arcturus Bioscience, and two descendents of the invention — PixCell and Veritas — are available from Molecular Devices of Sunnyvale, California. PixCell is a simpler, microscope-based platform for the manual dissection of cells, whereas Veritas offers a fully automated alternative for performing LCM. Both systems use a gentle near-infrared laser to partially melt an adherent layer of polymer film over selected cells; these cells can then be mechanically transferred with the film for analysis or further culture. Veritas also features a more powerful ultraviolet laser for rapidly cutting larger groups of cells or working with more difficult tissues or live cells.

Laser microdissection systems from Molecular Machines and Industries (MMI) of Glattbrugg, Switzerland, use a 'sandwich' approach in which cells or tissue samples are prepared between a layer of microscope slide glass and a polymer membrane; selected sections are cut with a brief laser pulse and then recovered with specialized collection tubes with adhesive caps. "The big advantage is that it's totally contamination free," explains Stefan Niehren, a senior development engineer at MMI. MMI offers two systems, the smaller and simpler SmartCut, and the CellCut, which offers full automation and can be expanded by integration with other MMI components, such as the CellManipulator optical-tweezers system.

Optical trapping and manipulation is also a feature of the CombiSystem, one of the PALM microlaser systems made by Carl Zeiss of Bernried, Germany. PALM systems use a non-contact process called laser microdissection and pressure catapulting, in which cells or tissue sections are precisely cut with a UVA laser and then catapulted into a collection vessel by a single beam pulse. PALM systems can be integrated with Zeiss microscopes and software for automated recognition and isolation of individual cells. "There are studies out now in which the PALM system was used to isolate an individual cell's clonal expansion, or to separate single embryonic stem-cell clones from other clones," says Richard Ankerhold, managing director of Zeiss subsidiary PALM Microlaser Technologies.

Leica Microsystems of Wetzlar, Germany, is another imaging specialist offering a platform for laser microdissection, the LMD6000, which has a powerful diode laser for precise cutting of thicker tissue samples and comes integrated with an automated research microscope.

Although these systems were first developed with fixed tissues in mind, most will also work with live cells, an area Liotta believes will grow in importance. "The exciting thing will be to actually do this with embryonic tissue or biopsies of living tissue from surgery," he says. "We'll see advances in molecular staining, stabilizing and extracting tissue macromolecules and being able to work with a thick piece of living tissue."

Increasing options

The longevity of the fluorescent cell sorter is a clear testament to its power, but subsequent years have also shown a need for complementary methods that can be applied for more specialized experiments — for example, sorting through dozens or hundreds rather than millions of cells, or plucking a handful of cells from a slice of brain tissue.

Meanwhile, growing interest in stem-cell isolation, clinical cell sorting and single-cell analysis are fuelling the drive to develop microscale cell sorters. Even though this field is still in its infancy, specialists in industry and academia are coming to recognize that microfluidic systems could one day handle many of the tasks now reserved for FACS. With their speed and proven reliability, it seems clear that modern cell sorters will remain cell-biology monarchs for some time — but they must also make room for what looks to be a growing court. ■

Michael Eisenstein is technology editor for *Nature* and *Nature Methods*.