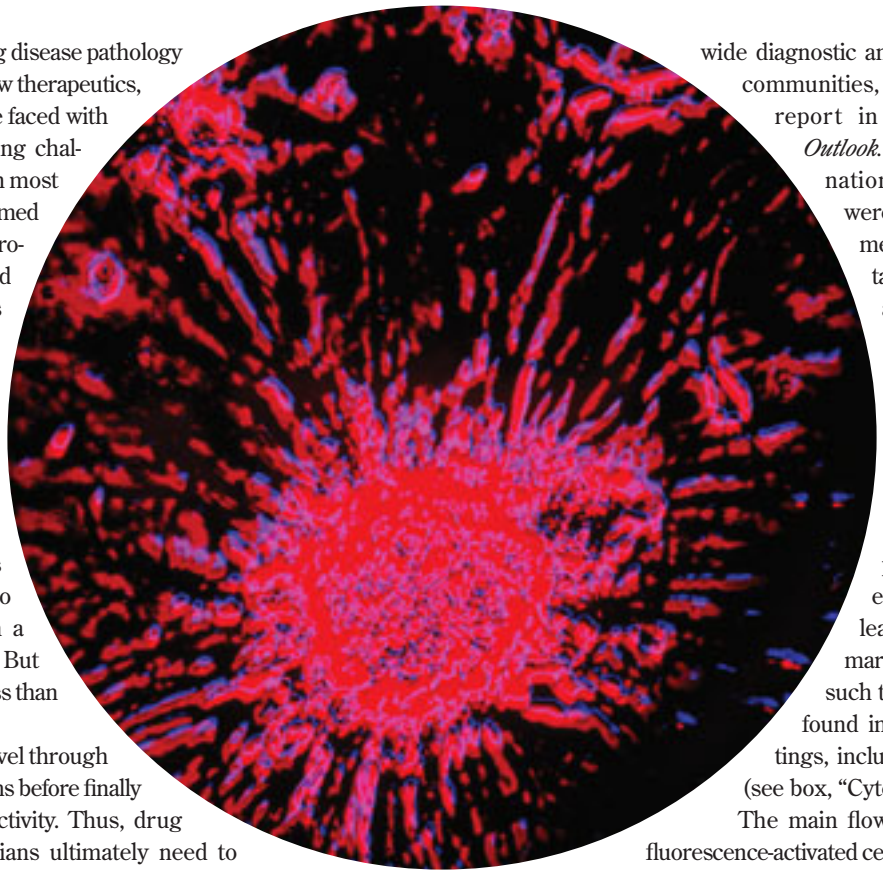
Sorting out the mess

Researchers are using a variety of methods to isolate specific cell types.

BY RANDALL C. WILLIS



PHOTODISC

In understanding disease pathology or designing new therapeutics, researchers are faced with a rather daunting challenge. Although most experiments are performed in vitro with isolated protein and/or nucleic acid systems, pathogenesis and drug activity ultimately occur in the context of an organism's cells. To address this problem, several research groups and companies have devised cell-based assays that allow scientists to test their theories in a more natural setting. But even this situation is less than optimal.

Drugs typically travel through a variety of body systems before finally finding their site of activity. Thus, drug developers and clinicians ultimately need to characterize drug behavior at the whole-organism level. This then brings the problem full circle, as researchers are left trying to ascribe the activities they see in assays to individual tissues or cell types. They need to know whether the effects they detect are global (organism-wide) or local (cell-specific).

To accomplish this, researchers have devised techniques to sort individual cells or cell types from a surrounding mélange of tissues on the basis of biomolecular characteristics.

Glow with the flow

Perhaps the oldest—but still predominant—of these techniques is flow cytometry, which represents an $860 million market in the worldwide diagnostic and life science research communities, according to a recent report in *Instrument Business Outlook*. In their earliest incarnations, these instruments were large pieces of equipment designed for hospital and industrial settings, and the market was dominated by three players: Partec, BD Biosciences, and Beckman Coulter. But over the past decade or so, technology improvements, steadily decreasing footprints, and new market entrants have joined the leaders in opening niche markets for flow cytometry, such that the instruments are found in many life science settings, including mobile operations (see box, "Cytometry on the road").

The main flow cytometry method is fluorescence-activated cell sorting (FACS), where researchers label specific cells within a mixture using fluorescently tagged antibodies that bind selected cell-surface molecules (Figure 1). They then deliver the cells into a thin stream so that cells pass singly through an exit nozzle, which vibrates to produce droplets containing individual cells. As each droplet passes through a laser beam, a computer registers its fluorescent properties, and if it meets predetermined criteria, it is given a mild electrical charge that varies depending upon which antibody it carries. The charged droplet then passes between a pair of charged metal plates, which deflect the droplet's flow to collection tubes or into a waste container.

Although researchers have applied FACS to a variety of cell-based assays, one area in which the technique is gaining use is drug screen-

ing and lead identification. For example, researchers at Imperial College London, the University of Bath, and pharmaceutical start-up Sterix (now a subsidiary of Ipsen) used FACS to monitor the effects of sulfamoylated estrogen derivatives on five prostate and ovarian cancer cell lines (*1*). They found that within 24 h of exposure to the drugs, each of the cell lines arrested in the G2/M phase of the cell cycle, signaling that the cells has ceased to proliferate. They also found that over the next 24 h, FACS peaks representing cells in the G1 phase became more prominent, suggesting the possible onset of apoptosis. Furthermore, whether they exposed the cells to the drugs continuously or washed and incubated treated cells in fresh, drug-free medium, the researchers found that the drugs' effects were irreversible.

"From these studies, it is apparent that 2-substituted estrogen sulfamates are emerging as a potent new class of drug that may be effective against androgen receptor positive and negative prostate and ovarian tumors in vivo," the researchers wrote. "Furthermore, they are also effective in ovarian cell lines that are resistant to known anticancer agents, indicating that the 2-substituted estrogen derivatives may also have a role in the treatment of tumors that have become resistant to conventional chemotherapeutic regimens."

Similarly, scientists at the biopharmaceutical company Aponetics use FACS as a second component of their screening program for drugs that promote apoptosis in cancer (*2*). In the first stage, they introduce a green fluorescent protein expression system into cancer cells and then monitor the effects that different known and potential drugs have on cell fluorescence. Whereas healthy cells glow brightly, cells undergoing apoptosis or necrosis begin to dim. In the second stage, the researchers use FACS to identify the drugs that specifically trigger apoptosis, and then complete the characterization using other assays, such as nuclear fragmentation, mitochondrial membrane potential, or cell cycle analysis.

Using this process, Aponetics has screened more than 60,000 compounds in a small-molecule library and has identified more than 17 lead compounds. The company recently initiated preclinical trials of five of these potential drugs, examining their activity in reducing human tumors in xenograft mouse models.

Mainly about magnets

For all of its success, however, FACS is not without its deficiencies. In several cases, intrinsic cell fluorescence has limited researchers' ability to distinguish positive and negative signals. Likewise, many researchers have expressed concerns about the cell throughput of typical FACS systems.

To surmount these problems, an increasing number of labs have started to apply immunomagnetic cell sorting (MACS) methods to isolate specific cells from a mixture. Like FACS, MACS relies on antibodies to label cells, but in this case, the antibodies are attached to biodegradable superparamagnetic beads that range in diameter from several micrometers to tens of nanometers. Thus, rather than rely on a laser to identify and an electrical charge to separate appropriately labeled cells, scientists use a powerful magnet to hold cells in the reaction chamber while washing away unlabeled cells. Researchers then collect the cells by turning off the magnetic field.

> "Gene expression profiling of highly purified cell subpopulations represents a major advantage over whole blood cell samples."

Recently, a group of researchers, led by Bernhard Gerstmayer of Memorec Biotec (a subsidiary of Miltenyi Biotec), used MACS to characterize the gene expression patterns of immune cells in people with ankylosing spondylitis (AS), a chronic inflammatory rheumatic disease of the spine and lower joints (*3*). Early efforts using whole blood identified several genes that showed differential expression, but it was difficult to determine whether the results represented true disease-related regulation patterns or if they were simply a product of differences in relative cell numbers in the samples. To address this question, Gerstmayer's group isolated CD14+ monocytes from peripheral blood samples taken from AS patients and healthy donors. They then isolated RNA from these cells and examined the gene expression patterns using PCR amplification and microarray detection.

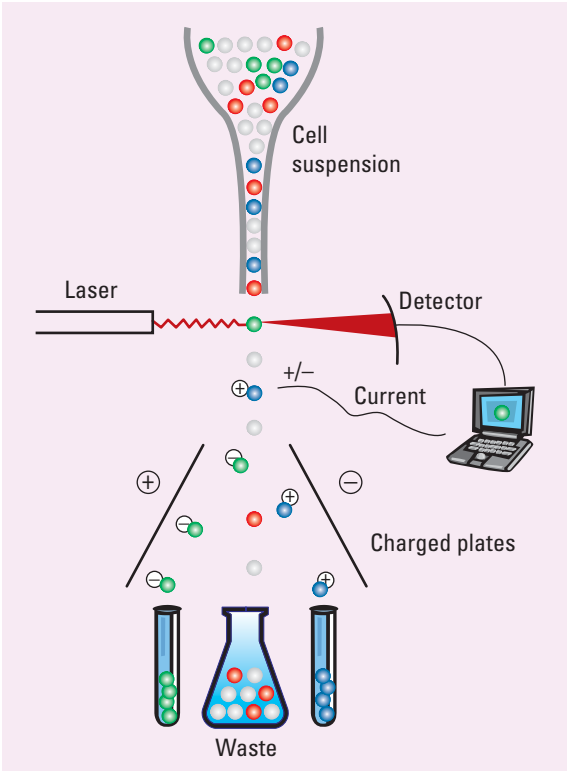


Figure 1. Just the FACS. A schematic of fluorescence-activated cell sorting.

The researchers determined that MACS produced cell populations that were 98% pure and observed a strong correlation between gene expression levels and CD14 status. Using 2D cluster analysis, they found that 8 of 11 AS patient samples could be grouped into a separate cluster from healthy control individuals. Likewise, they identified many genes that were exclusively regulated (up or down) in AS patients but not in healthy individuals. These findings, they suggest, show "gene expression profiling of highly purified cell subpopulations represents a major advantage over whole blood cell samples."

On the drug discovery side, researchers at Hiroshima University used MACS to examine the impact of neurosteroids on the biological pathways involved in glaucoma pathogenesis (*4*). Earlier research showed that glutamate concentrations in the eyes of glaucoma patients were double those found in healthy people, and that excess glutamate induces apop-

tosis of retinal ganglion cells (RGCs). Thus, the researchers wanted to see if RGCs carried glutamate receptors and whether neurosteroids that modulate the function of one glutamate receptor subtype might inhibit glutamate-induced neurotoxicity in RGCs.

Using MACS to isolate rat RGCs and patch-clamp methods to identify receptor activity, the researchers found that cells carried three varieties of glutamate receptors. Furthermore, they were able to potently inhibit the activity of one receptor subtype using the neurosteroid 20-hydroxyecdysone, but they were unable to determine whether the drug worked directly on the receptor or indirectly through secondary pathways. Regardless, they were confident that these findings offer a lead in exploring ways to inhibit glutamate-induced RGC death and possibly treat glaucoma.

MACS is also not without its problems, however, as using paramagnetic beads limits researchers' ability to perform multiplex reactions, unlike FACS, where they can use different fluorophores. Thus, in many cases, researchers use a combination of MACS and FACS to achieve their goals.

Moving to microchips

Because techniques such as FACS can suffer from large sample requirements, high background fluorescence, and the potential for cross-contamination, numerous research groups have looked to microfluidic and materials science technologies to address some of these issues. For example, Stephen Quake and colleagues at the California Institute of Technology developed an integrated microfabricated cell sorter using soft lithography (*5*).

The cell sorter has two layers: a top piece that carries pneumatically controlled channels for the pumps and valves, and a bottom layer that carries the microfluidic lines (which form a T shape) for sample injection, collection, and waste removal. The sorter is placed on an inverted microscope, and a laser is used as the excitation source. A photomultiplier tube detects the fluorescence of passing cells.

As the cells move along through the sorter, they pass through the fluorescence detector at the junction of the three sample lines. If no signal is detected, the pumps send the cell to the waste channel. If a cell fluoresces, however, the buffer flow is reversed, which sends the cell back through the detector. If fluorescence is detected a sec-

Cytometry on the road

While most people in the developed world think little of clinical instrumentation, fully expecting their local hospitals to be equipped with the latest and greatest technologies, life is different in large segments of the developing world. In extensive regions of sub-Saharan Africa and Central Asia, areas that have been devastated by the effects of diseases such as HIV/AIDS and malaria, most people live well removed from large cities and have access to few or no hospitals with high-technology infrastructures. For this reason, clinicians and aid workers have been forced to take the medicine to the people as opposed to bringing the people to the medicine.

"With many resource-limited areas of the world now gaining access to more affordable antiretroviral therapies, simpler and less costly methods of monitoring treatment—including CD4 and CD8 T-cell counts—are urgently needed," said Barry Bredt, director of core laboratories at the General Clinical Research Center of the UCSF/San Francisco General Hospital, at the 15th International AIDS Conference in Bangkok in July 2004.

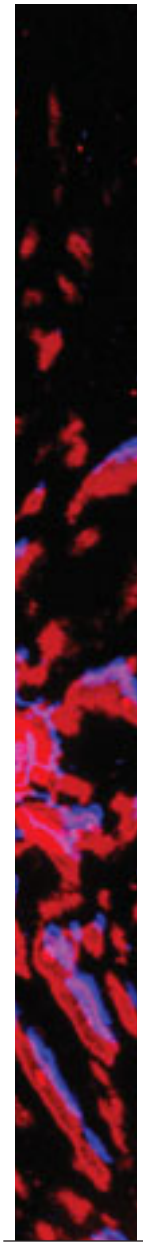
At the conference, Bredt and other researchers described their studies of the effectiveness of Guava Technologies' EasyCD4 and EasyCD8 cytometry assays for monitoring the immune status of HIV patients. The assays rely on integrated systems of reagents, software, and microcapillary instrumentation that allow them to be compact, portable, and low-maintenance. In preclinical studies, the researchers found the miniaturized assays, which require only 10 µL of blood, offered results similar to those obtained using traditional, clinically approved, and more expensive flow cytometry tests. Given these results, Guava intends to seek U.S. FDA approval for use of the assays as clinical diagnostic products.

Similarly, Roland Göhde and colleagues at Partec have developed a mobile cell-sorting lab—CyLab—that functions out of the back of a truck, powered by a 12-V car battery or solar panels. Working with clinicians from around the world, Göhde, Partec's HIV/AIDS project coordinator, travels to remote regions to test the immune status of HIV-positive patients. In particular, they monitor the number of CD4+ T lymphocytes, cells that are targeted and destroyed by HIV, in patient blood samples using flow cytometry. When patient cell numbers drop below 200 cells/µL of blood—normal levels are 435–1600 cells/µL—the patients are prime candidates for antiretroviral treatment.

PARTEC GMBH

CyLab: A mobile immune system monitoring station for HIV/AIDS patients.

According to a recent report by Göhde and Burkhard Greve, a radiobiologist at the University of Münster (Germany), CyLab technology has reduced the cost of a CD4 assay from $48 to $2.50 and dramatically increased the reach of clinical efforts in resource-poor regions (*7*).



ond time, the cell is then sent to the collection channel.

The researchers tested their system on a mixed population of *E. coli* expressing either enhanced green fluorescent protein (EGFP) or *p*-nitrobenzyl (pNB) esterase. After sorting, the cells in the waste and collection channels were plated onto nutrient agar containing either ampicillin (upon which the EGFP cells would grow) or tetracycline (upon which the pNB cells would grow). Almost 500,000 cells could be sorted in a single run, recovery yields reached 50% for some experiments, and cells were enriched up to 89-fold.

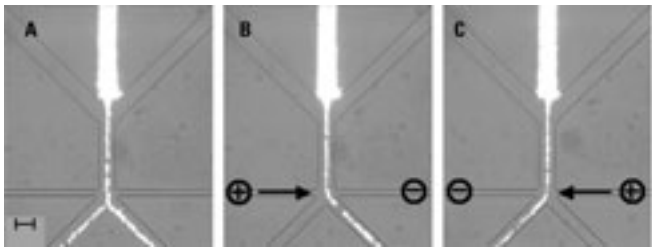


Figure 2. Microfluidic cell sorting. Under hydrodynamic flow, fluorescent particles flow equally into the left and right lanes of a microfluidic cell sorter (A). Upon application of an electro-osmotic current, however, cells are deflected right (B) or left (C). (Reproduced with permission from Ref. 6.)

The size of the integrated cell sorter means that very small samples can be handled quickly and with reduced background fluorescence as compared to FACS. Furthermore, the detection optics offer superior sensitivity, and the simple fabrication and inexpensive materials make the unit disposable, eliminating problems of cross-contamination.

Petra Dittrich and Petra Schwille from the Max Planck Institute for Biophysical Chemistry have developed a similar microfluidic cell sorting system that researchers can use to perform integrated reactions, detection, and isolation (*6*). In this case, however, rather than simply rely on pressure-driven buffer flow to push cells through the system, the researchers incorporated brief pulses of electro-osmotic force to deflect the cell stream into waste and hold reservoirs (Figure 2).

Using fluorescence correlation spectroscopy to detect the tagged cells and a Y-shaped microfluidic chamber, the researchers could identify single cells easily and deflect them into the appropriate stream with 80–95% efficiency. To improve this statistic, they propose adding subsequent selection steps in line. According to the researchers, "The future potential certainly lies in the highly specific, ultrasensitive, and fast screening of large libraries of cells, cell organelles, aggregates, and small fluorescent particles, yielding information about the distribution of subpopulations."

Thus, regardless of the targeted application or the biophysical methods used for selection or detection, researchers are finding new and subtle ways to isolate individual cell types from heterogeneous populations. And throughout the developmental history of these techniques, demands for higher throughput and lower costs have led to microfluidic manipulations with ever-shrinking samples.

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