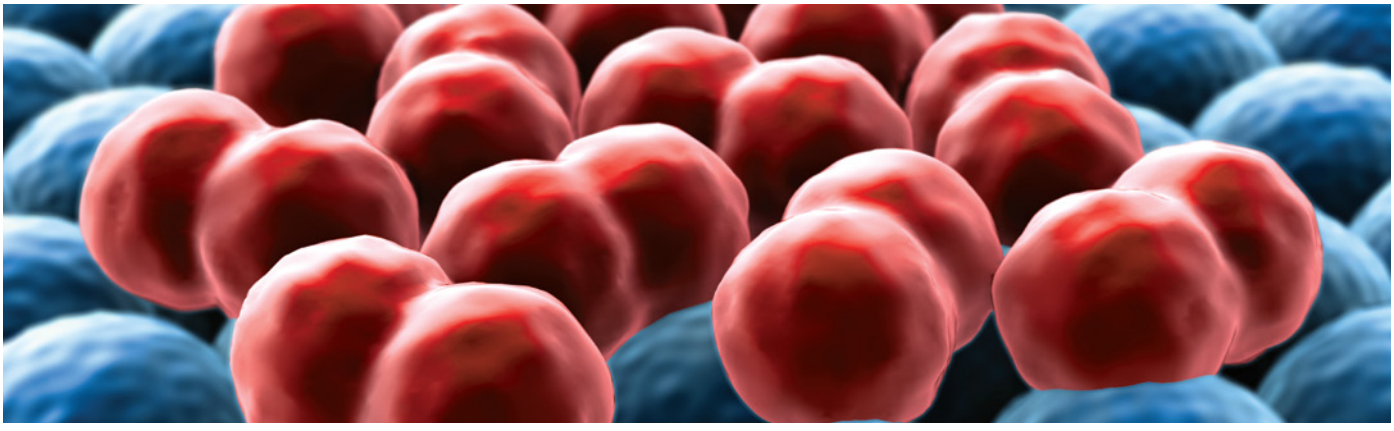

Cell biology shapes up

Cell biologists no longer rely only on pictures from microscopes—pixels and pulses from cell sorters and other devices now enhance their research. By borrowing tools and techniques from computer scientists and engineers, they have upgraded their approach to studying cell morphology.
By Alan Dove

The shape we're in

Since its inception, cell biology has been a fundamentally visual science. Fibroblasts are elongated; neurons are dendritic. Mitotic cells have spindles; apoptotic cells have blebs. The field's clinical cousins—pathology and hematology—also rely heavily on cell shape. Pathologists classify tumors by how far their cells deviate from their normal forms, while hematologists identify and count different leukocytes by sight.

This visual approach has driven decades of crucial discoveries and decorated countless labs with stunning wall art, but it's also revealed the limits of traditional techniques. To push the field further, cell biologists have begun borrowing machine-learning and data-mining tools from computer science, while engineers are designing imaging and microscopy systems that enable entirely novel analyses.

At the same time, biologists are gaining a better appreciation of the importance of cell morphology. Although researchers have long known that genetic and epigenetic mechanisms can alter cells' shapes, recent work has shown that the reverse is also true. Now, a combination of innovative 3D culture systems, automated image analysis, and sophisticated microscopic shape mapping is driving a rush of groundbreaking discoveries.

Faces in the crowd

For immunologists and hematologists, cell morphology research has enjoyed decades of steady progress. Since the development of the earliest clinical hematology analyzers, automated cell counting based on cellular shapes has been a staple technique of medical and basic research laboratories. Fluorescence-activated cell sorters added the ability to separate cells into containers based on which molecular markers they carry.

More recently, engineers have added complete imaging capability and artificial intelligence to some of these machines, yielding systems that can simultaneously photograph and classify thousands of cells per hour. "They aim not just to quantify markers … you're actually able to localize [markers], look at them, and make deductions from the cell shape or the nuclear shape overall," says Shaf Yousaf, head of technology and business development at **MilliporeSigma** in Billerica, Massachusetts. He adds that "they can do all the things classical flow cytometers do, as well as look at images of individual cells."

The MilliporeSigma systems produce immense quantities of data, which is both a blessing and a curse. Because no human could reasonably sift through thousands of cell images to distinguish relevant morphological differences, the machines rely heavily on image analysis software. Depending on which device they buy, researchers can photograph cells at anywhere from 20x to 60x magnification, and use the included software to sort them by purely visual characteristics such as shape and size, fluorescent markers, or both.

Automated image analysis enables studies that would be difficult or impossible with traditional microscopy. One MilliporeSigma system user is developing label-free assays to distinguish blood cancers. "When you look at the varying types of cell morphologies that correspond to different leukemias, you can statistically look at enough cells using this system to be able to classify different kinds of leukemias," says Yousaf. The ability to track markers inside and outside a cell simultaneously, and to map them atop images showing the cell's shape, is also helping researchers study the metabolic effects of signaling molecules, such as G-protein-coupled receptors.

MilliporeSigma sells a range of options with its imaging flow cytometers, but whether an individual laboratory is buying a low-end unit or a core facility is installing a more complex one, researchers should expect to spend some time learning how to use these instruments. The

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bulk of the training focuses on experimental design and data analysis. Typically, scientists use a set of manually validated cell images to "train" the software's learning algorithms on the desired morphological traits. They then run samples through the system, see whether the machine is identifying the correct shapes, and retrain the algorithm as necessary.

Enter the matrix

While blood cells live in a convenient, easily studied liquid, most other cell types have been harder to analyze in the laboratory. That's why cell biologists were justifiably fascinated when 2D cell culture first became available. Growing immortalized cells on petri dishes provided unprecedented control over experiments, but at a cost. "Of course we learned quite a bit from that technique, but at the same time they were also using completely [artificial conditions]," recalls Mina Bissell, a distinguished scientist in the Biological Systems and Engineering Division at **Lawrence Berkeley National Laboratory** in Berkeley, California.

During her postdoctoral work, Bissell noticed that removing mammary cells from a mouse and putting them into a culture dish caused them to lose not only their normal in vivo shapes, but also their ability to secrete milk. "I said 'Hey, why don't we put these things in something that looks like a basement membrane?'" says Bissell. That idea led eventually to a 3D culture system, in which isolated mammary cells retained their shapes and also their milk-secreting ability. Since then, Bissell and a growing number of colleagues have embraced the idea that for solid tissues, cell context and shape are at least as influential as genes.

"Signaling pathways and gene expression profiles are very different in cells that grow in 2D and 3D cultures," says Oksana Sirenko, a research scientist at **Molecular Devices** in Sunnyvale, California. Besides displaying more natural signaling patterns, 3D cultures allow researchers to mix cell types in ways that mimic their interactions in living tissue, providing more accurate models of normal physiology and pathogenesis. As a result, 3D cell culture systems have become a staple of cell biology. "3D culture is trying to bridge a gap between in vivo biology and in vitro cell biology," says Sirenko.

In a typical 3D culture system, cells cling to each other, forming spheroids that float in liquid culture media or expand into a soft matrix. Investigators can watch the cells change shape as these spheroids grow and respond to stimuli. For example, researchers can grow spheroids that mimic early-stage tumors, then treat them with candidate chemotherapies and watch for the spheroids to stop growing or start shrinking. Tracking such changes manually, however, is slow and subjective.

"[The] challenge is how to get reliable information and do it in a more high-throughput and more reproducible manner—so here comes technology," says Sirenko. Companies making high-content cell imaging equipment such as confocal microscopes and plate readers are now adding software tools to process images of 3D cell cultures. In a typical algorithm, the software first scans each image for spheroid-like structures, then defines the boundaries of likely cells within the spheroids. Researchers can query the data to score how many live and dead cells the spheroids contain in each plate or well. They can also track standard molecular markers for mitochondria, nuclei, and other subcellular structures. "You can not only evaluate changes such as cells dying, for example, but you can also see more subtle phenotypic changes between different [conditions]," adds Sirenko.

Molecular Devices integrates this type of software into high-throughput screening platforms, so scientists can quickly scan thousands of spheroids growing in varied culture conditions. The system can yield dose-response curves for candidate drugs, screen the phenotypes of large numbers of gene mutations, or execute complex custom protocols for completely new experiments. Despite the heavy automation, though, Sirenko emphasizes that researchers retain the ability to evaluate the cells manually: "I can check any number or I can check an entire plate and see whether it [matches] my visual assessment." Other companies offer similar tools on their own high-content screening platforms, such as **GE Healthcare Life Sciences'** IN Cell Analyzer system.

Pharmaceutical companies are one obvious market for high-throughput cell morphology equipment, but competition and rapidly increasing computing power have now driven prices down to levels many academic users can afford. Sirenko estimates that basic research laboratories now comprise about half the users of these systems.

You know it when you see it

The ability to automate morphology assays on 3D cell cultures is a powerful tool, but some scientists are now pushing the technology even further, with algorithms that can classify structures in living animals and clinical specimens. Those analyses have long relied on qualitative changes in cell shape to track physiological shifts. For example, neurobiologists can track the activation of microglia, the brain's native immune cells, based on their shapes. "It was well known for decades that activated microglia retract these very branched filopodia, and they become round," explains Cleo Kozlowski, a scientist at **Genentech** in South San Francisco, California. cont.>

However, that assay doesn't pinpoint the moment of activation or provide a straightforward way to quantify the connections between microglial activation and gene expression patterns. Kozlowski tackled that problem computationally; using the popular MATLAB programming environment, she and her colleagues developed an algorithm that classifies the shapes of microglia in confocal microscopy images with 90% accuracy. The technique works in sectioned brain tissue, and also in the brains of live mice with transparent windows implanted into their skulls. By quantifying the cells' shapes, the algorithm allows researchers to determine precisely how far along the activation pathway they are. Overlaying that information onto data from gene-expression profiling characterizes the microglia with unprecedented precision. Since publishing the technique in 2012, Kozlowski says she has provided the code to numerous researchers on request.

More recently, Kozlowski has focused on diagnosing colitis. "Colitis is generally assessed by a pathologist, so they have to sit down, look at various areas under a microscope, [and] score how bad the inflammation and the morphological changes of the crypts are in the intestines," says Kozlowski, adding, "I just wanted to speed up that process."

Pathologists typically use hematoxylin and eosin (H&E) staining to highlight the intestinal structure. Unfortunately, this type of staining is hard for computers to interpret. "It doesn't stain a specific molecule of interest—it gives you a kind of overview of the tissue structure, so it's really about morphology and shape," says Kozlowski. She compares fluorescence microscopy to searching an image of the night sky for stars, whereas using H&E is more like picking out cities on Google Maps, a much harder computational problem.

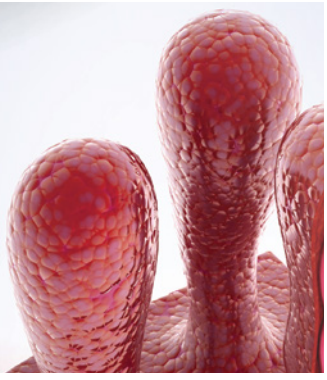
To make the analysis work, Kozlowski and her colleagues built their algorithm inside a powerful, proprietary image-processing framework from **Definiens** in Munich, Germany. The result was a system that can distinguish healthy intestine from colitis quickly and accurately, but at a price only very well-funded corporate researchers can afford—the Definiens license is prohibitively expensive for most laboratories.

The major challenge for automated pathology assays, though, may be cultural. "That will be the biggest hurdle, really convincing a lot of people who don't really think that this kind of thing can be done by machines to say, 'Oh, yes it can,'" explains Kozlowski.

Look and feel

Besides devising unique ways to analyze conventional cell images, researchers are also using new microscopy techniques to better define cell shapes. In atomic force microscopy (AFM), a silicon probe scans across a surface and builds a 3D image of the cells it encounters, much like someone reading a relief map with their fingers. AFM is especially useful for identifying changes in the cytoskeleton and studying relatively hard surfaces such as bone.

"AFM didn't catch the details of microvilli and other structures on the cell surface. If we adopt [SICM], we can actually open a new chapter in cell biology."
—Sang-Joon Cho



For soft cells, though, AFM yields fuzzier images. "Looking at live cell membranes is almost impossible, because the cell membrane is not a simple structure; there are a lot of glycoproteins as well as the extracellular matrix," explains Sang-Joon Cho, chief scientist and director of R&D at **Park Systems** in Suwon, Korea. The silicon probe tends to squash the membranes of soft cells while measuring them, deforming many of the structures that cell biologists are most interested in seeing.

To address this problem, Cho and his colleagues turned to scanning ion-conductance microscopy (SICM). Instead of a solid silicon probe, SICM squirts a stream of saline solution through a thin capillary tube. Measuring changes in a voltage bias applied to the saline reveals how close the capillary tube is to the cell, allowing researchers to scan subtle structures on the cell surface without disrupting them.

For Cho, the results were revelatory. Comparing images of embryonic stem cells made with AFM and SICM, he found that the SICM images revealed things that had been invisible under AFM. "AFM didn't catch the details of microvilli and other structures on the cell surface," says Cho, adding that "if we adopt this technology, we can actually open a new chapter in cell biology."

Park's SICM systems also have AFM and optical microscopes built in, so scientists can view cells with all three techniques and compare or superimpose the resulting images. The systems can also enhance other techniques, such as patch clamp measurements applied in electrophysiology. Investigators can use SICM to pick a specific point on the cell membrane, then attach the patch clamp there to measure the membrane's electrical potential. They can also deliver precise doses of drugs, nucleic acids, or proteins to targeted points on the cell.

Both AFM and SICM require specialized training, but most biologists find the latter instrument easier to learn. "When I train people, they take several months to get certain images from soft biological samples using atomic force microscopy, but with SICM they can get them in a week," says Cho.

Regardless of the approach they take, experts in cell morphology agree that the field is poised for a major leap. "The two keys for me moving forward are machine learning and better optics," says Kozlowski, adding, "it's a very exciting time in image analysis."

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