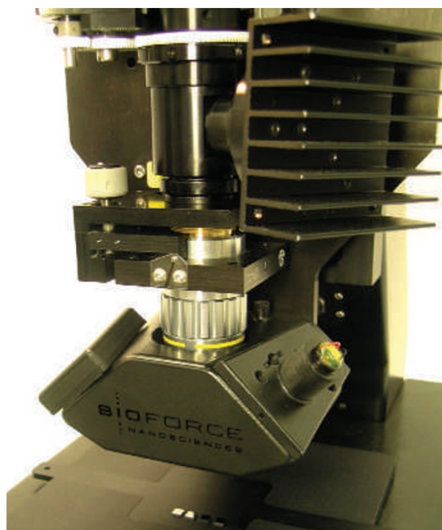
Close-up on cell biology

To define the workings of cellular structures and molecules requires cutting-edge technology not only in biology and biochemistry, but also now in nanotechnology. Hayley M. Birch and Julie Clayton report.

The dawn of the nano-era has placed a whole new array of tools in the hands of cell biologists who are keen to go deeper into the intricacies of how cells work. Forever pushing the boundaries, cell biologists are shifting focus from the micro- towards the nano- and even sub-nano level. To do so means having to find ever more creative ways of using not just biology, but also elements of physics, materials science and engineering. Biophysicists, nanotechnologists, nanofabricators and electrical engineers are working side by side to probe further into the cell.

Nanotopography

Knowing what makes a cell tick is as much about understanding its outside environment as its internal workings. The availability of sophisticated nanopatterning and nanotopography techniques — in which surfaces can be etched and coated with a variety of substances at the nanoscale — is enabling cell biologists to gain a more precise understanding of, and control over, cellular responses compared with cell culture in conventional glass or polystyrene culture plates A range of substrate structures designed specifically for exploring cell behaviours such as spreading, migration and adhesion are under development (see "Cell culture in three dimensions" and "Down to the letter", page 940).



The Nano eNabler deposits molecules at nanometre resolution.

BIOFORCE

The field is so new that most research teams are creating their own nanopatterned surfaces on which the cells are cultured. At Columbia University, New York, Michael Sheetz is developing nanopatterning methods with a team of cell biologists, systems biologists and nanofabricators. "We put down arrays of different spacings of molecules and use those to measure cellular responses," he explains. "We have found that the spacing really matters." Sheetz and colleagues are currently using patterning to examine how the spacing of protein ligands on a substrate surface is important in the binding of dimeric proteins such as talin.

While Sheetz and many others are currently using home-made devices, commercial developers are beginning to sense an opportunity. BioForce Nanoscience in Ames, Iowa, has developed the Nano eNabler, a device that can deposit molecules on surfaces at defined locations at nanometre resolution, and which, among its various options, can be used to apply extracellular matrix proteins onto cell-culture surfaces in predefined patterns. A key feature of the machine is that the drops deposited can be as large as 100 μm or as small as 2 μm

CELL CULTURE IN THREE DIMENSIONS

Taking the idea of nanoscale design for cell culture further, some cell biologists are investigating cellular responses to more complex three-dimensional structures.

A collaboration in this field between cell biologists and electronic engineers at the University of Glasgow's Centre for Cell Engineering is proving fruitful.

The group has the advantage of a state-of-the-art electron-beam nanolithography system (E Beam) manufactured by Leica, which creates nanoscale features on a surface under the control of customized software (see also 'Down to the letter'). "We're quite unique in the United Kingdom in that we have access to such an incredible machine with which you can define structures so deliberately," says Mathis Riehle, who directs research at the centre. "With the E Beam you can be very specific."

Mathis Riehle: "With the E Beam you can be very specific."

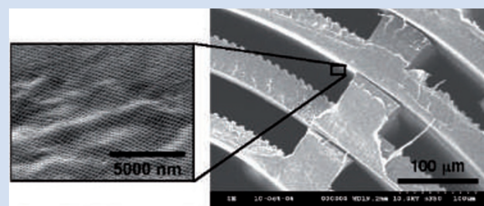
The E Beam is used to define or "write" a pattern at nanometric resolution into an electron-sensing polymer. The pattern is developed and used as a template for etching or depositing material to form the desired nanotopographic features. This technique gives a greater degree of control than 'natural' lithography, which relies on self-assembly of colloidal particles in regular arrays to produce the pattern.

Riehle wants to explore the limits of the machine. "We've made tubes and we want to make more complicated three-dimensional structures — maybe structures that will look like cell 'car parks'. But to do that we would need something like origami, because at the moment the E Beam can only really write on a flat plane. We cannot write on a shaped, undulating surface." The team will have to write its own software to create these complex structures.

As one of a consortium of institutions working on emerging nanopatterning methods, the Glasgow centre is also developing alternative methods of engineering 3D structures with nanoscale features for use as cell-culture substrates.

Kris Seunarine and Osian Meredith have fabricated a 'swiss roll' from e-poly-caprolactone, a biodegradable thermoplastic shaped by hot nano- and micro- embossing. This is the 'sponge' of the swiss roll, with the cells as the 'jam'.

Two levels of microfeatures are sculpted into the polymer (see photos). One aligns fibroblasts and smooth-muscle cells, keeping them from being squashed. The inward-facing surface is patterned at the nanoscale with a regular array of pits (100 nm diameter and 80 nm deep) spaced 300 nm apart, which define locations for endothelial cell adhesion. These cell scaffolds could be useful in developing methods for vascular and urogenital reconstruction. H.M.B.



K. SEUNARINE & O. MEREDITH

Cell support: ridges on the surface of the 'swiss roll' will keep cells aligned, and pits provide anchorage.

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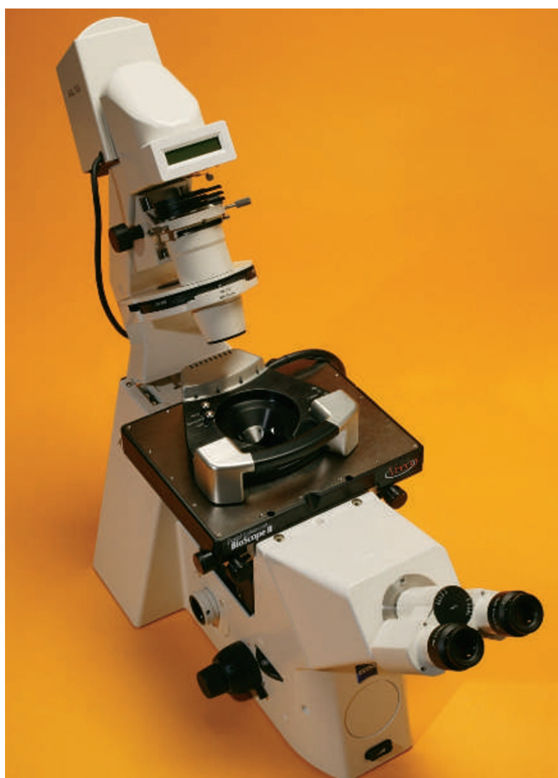

Two in one: the BioScope II combines atomic force microscopy with fluorescence microscopy.

across — smaller than a single cell. "The cells can interact with many spots at once, which enables the investigator to construct more complex questions, such as how well a cell will respond given a choice of two different proteins," says BioForce's product manager Michael Lynch. The fine control is achieved through a microfluidic cantilever, which dispenses liquid onto a surface that is etched into defined regions.

The Nano eNabler was originally developed for the biosensor market, to look at interactions between proteins, DNA and RNA without the need for labelling. But interest has grown in the past year from researchers wishing to use the device for nanopattern-based cell culture. Around a dozen research groups are currently exploring its potential for this kind of work, including the study of neurons.

Lynch anticipates that in future cell biologists will prefer to use the equipment to design their own nanopatterns rather than buy nanopatterns off-the-shelf. "There are so many different questions being asked that it's hard to narrow down to one pattern, chip or substrate for cell culture. It's not as standard as the microarray field, which has turned into a service."

Two worlds collide

Until recently, cell biologists using confocal microscopy and those using electron microscopy (EM) inhabited two completely separate worlds: confocal microscopists made movies in glorious technicolour, whereas EM practitioners produced black-and-white stills. Both caught cells on camera, but neither got all the results they really wanted.

For confocal film-makers the problem is the lack of resolution, as this has to be sacrificed in order to capture live-action cell behaviour. EM experts, on the other hand, have all the resolution they require, but only a slim chance of snapping a cell in the process of doing something interesting.

But at last these two worlds are slowly converging to give correlative light and electron microscopy through a new and surprisingly simple technology. Ian Lamswood, marketing manager for Leica Microsystems in Vienna, Austria, explains how Leica was approached by Paul Verkade and his colleagues from the Max Planck Institute for Cell Biology in Dresden, Germany. "They wanted to look at the same cell in the EM as they had in the confocal; basically to freeze-frame it and look at the same proteins being synthesized at the same moment in time."

Verkade, an electron microscopist now at the University of Bristol, UK, recalls, "I saw a paper in *Nature Cell Biology Reviews* that said it would be great to have something that could combine fluorescence and electron microscopy. It was a really funny coincidence because we were working on it, but of course, I couldn't say." His invention, currently marketed by Leica as the Rapid Transfer System (RTS), takes a sample from under the confocal microscope to a high-pressure freezer within a few seconds, in preparation for electron microscopy. The RTS has been designed as an attachment to Leica's EM PACT high-pressure freezer. It consists of a rapid loader, which holds the live sample beneath the confocal, then transfers it quickly to the high-pressure freezer, creating a specimen that can be used for EM. Confocal microscopy allows the user to pinpoint a potentially interesting cell event, while the high-pressure freezing preserves the fine ultrastructure at the chosen moment. This provides the researcher with a snapshot of a known cell activity. Using the RTS system, the time for transfer between light microscopy and the fixation of a cell for EM is reduced from around a minute to less than 5 seconds. The state of the cell viewed in the EM is then the same as that last seen by the confocal microscope, but at a much higher resolution. "This machine has the added advantage that it is really good for standard fixation for EM," notes Verkade.

Paul Verkade: solving the problem of combining fluorescence and electron microscopy.

For other investigators, the most appealing new development is the combination of atomic force microscopy (AFM) with fluorescence microscopy. Veeco, of Woodbury, New York, has combined AFM with an inverted optical or confocal microscope in the BioScope II, which enables the high-resolution images of AFM to be complemented by fluorescence microscopy. The BioScope II is an advance over previous Veeco models, with new software for greater flexibility and control by the investigator.

Dennis Discher and his team at the University of Pennsylvania in Philadelphia are using the BioScope II for investigating the differentiation of stem cells on different types of substrates. In particular, they want to assess the rigidity of the cytoskeleton and mechanical changes in its microenvironment at the various stages of differentiation. AFM, like scanning EM, gives highly precise topographical information — what Discher calls "blobology" — such as height, length and shape. But in contrast to EM, AFM can be performed on live cells. Combining this with a fluorescence microscope allows investigators at the same time to identify and track the various structures observed by AFM, for example, in cells expressing proteins tagged with green fluorescent protein.

"You can do mechanical interrogations on cells, and on isolated molecules and complexes, that you can't do with EM — such as make force measurements — and as well you can treat with drugs, and then push and poke to characterize remodelling, and this can all be done before, during and after imaging by fluorescence," says Discher.

JPK Instruments in Berlin, Germany, also markets a new AFM instrument — the NanoWizardII BioAFM — which can be mounted either on a confocal laser-scanning microscope (such as the LSM 510, from Carl Zeiss in Jena, Germany) or on an inverted light microscope (such as the Zeiss Axiovert 200). This allows the user to identify the location of cells of interest across a wide field of view before using AFM to scan a particular region at high resolution and/or perform fluorescence imaging. JPK Instruments also supply a cell-culture vessel (the JPK BioCell) in which cells can be maintained in buffer or cell-culture medium at 37 °C for the duration of the observation. The AFM tip can be coated with ligands or used to prod cells mechanically, and the ensuing biochemical reaction observed by fluorescence.

JPK's DirectOverlay software includes calibration that enables the user to integrate the various types of images and precisely superimpose a fluorescence image onto a 3D projection of a cell. These techniques are particularly

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well suited to studying the role of the cytoskeleton in processes such as cell spreading and attachment. Another company improving the performance of atomic-force microscopy by integrating it with confocal microscopy is Asylum Research of Santa Barbara, California, while nAmbition in Dresden, Germany, offers instruments for automated force spectroscopy.

Nanomanipulations

Going beyond observation to direct cell manipulation, Iva Tolic-Nørrelykke and her colleagues at the Max Planck Institute for Cell Biology in Dresden have brought optics giant Olympus in Hamburg and optoelectronics company PicoQuant, based in Berlin, together to market a combination of confocal microscopy and a picosecond (10^{-12} s) pulsed diode laser cutter, to enable manipulation of subcellular structures at the same time as viewing the results. "People have done laser ablation before but mainly with an ultraviolet laser on other microscopes. The new picosecond laser is simple and cheap, and induces less damage than UV because of its longer wavelength and short pulses," Tolic-Nørrelykke explains. She is using the device to examine the effects of laser cutting on the ability of microtubules to maintain the position of cellular organelles.

And taking the idea of combinations a step further, Tolic-Nørrelykke is also exploring, so far in the lab rather than commercially, putting together a two-photon microscope with a femtosecond (10^{-15} s) pulsed laser — to do both fluorescence imaging and laser cutting — and adding another, continuous, laser. This laser will act as optical tweezers, which can trap and displace microscopic objects. (Two-photon microscopes are a type of fluorescence microscope that enables imaging of living tissue down to a depth of 1 millimetre with low bleaching and photodamage.) This combination has enabled her team, for example, to specifically destroy small portions of the cytoskeleton and then use optical tweezers to displace the nucleus and follow how the cytoskeleton works to restore the correct position of the nucleus in the centre of the cell.

The Max Planck team has put together its system from standard commercial products from companies such as Zeiss, Hamamatsu and Thorlabs. "These techniques have existed for 10 to 20 years but we have optimized and combined them in one set-up," says Tolic-Nørrelykke.

Looking ahead, optical tweezers are likely to become an even more sophisticated tool for manipulating either whole cells or subcellular structures. This is being made possible through the use of holograms to split the optical tweezer laser into multiple beams, each one capable of directing the movement of a separate structure. By projecting a sequence of holograms, each positioned slightly differently to the one before, a sequence of light patterns occurs that can be made to move multiple objects around in three dimensions, in a kind of animated dance, according to physicist David Grier at New York University. Grier created the spin-off company Arryx in Chicago, Illinois, based on this concept, the result being BioRyx 200, a device that has a variety of applications, including sorting cells according to size, shape and composition, differentiating between cancerous and non-cancerous cells, and distinguishing bacteria from viruses.

MPI-CBG, DRESDEN

Iva Tolic-Nørrelykke: putting lasers and microscopes together in new combinations to manipulate cellular components.

Further developments in software and photonics means that holographically controlled optical traps will become an important technology for manipulating structures inside cells in the next 10–20 years, predict biologist Daniel Robert and physicist Mervyn Miles at the University of Bristol's new nanoscience centre. Robert and Miles collaborate with Miles Padgett at the University of Glasgow to explore the use of 'haptics' — computer-tracked hand movements — to manipulate objects through the movements of optical traps (see R. Webb, *Nature* **444,** 1017; 2006).

These developments will help cell biologists gain a different perspective. "The mechanical aspects of biology at the micro- and nanoscale have not been explored to their full potential. There are now lots of new tools that can enable us to do that," says Robert.

Nanoprobes

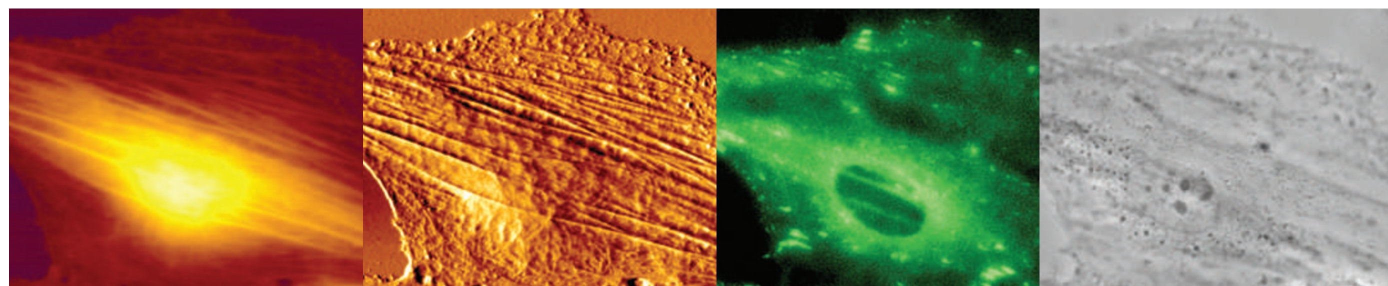
Nanotechnology is also proving useful to biologists wishing to focus on single molecules. Nanoprobes such as quantum dots, semiconducting crystals that can fluoresce in a wide variety of colours, work as cell spies, shadowing the movements of their molecular quarries and allowing the investigator to follow in-cell events on camera with an astonishing level of detail.

Such close camera work requires sophisticated imaging solutions. In confocal microscopy, the majority of photons emitted from a fluorescent light source are rejected, so as few as two out of every 100 falling onto a pixel are lost. Charge-coupled device (CCD) image detectors improve on this low level 'quantum efficiency' by capturing more photons and converting them into electrons, but what is really needed is a way of amplifying the signal above the level of background noise. Colin Coates, market development manager for Andor in Belfast, UK, explains how electron multiplying CCD (EMCCD) improves on standard CCD, "It's like trying to find something hidden in long grass," says Coates, "With EMCCD we amplify to such a high level that those photons aren't lost any more."

Having pioneered the technology, Andor now markets a range of EMCCD cameras for studying single-molecule dynamics. Stefan Diez, at the Max Planck Institute of Cell Biology and Genetics in Dresden, uses an Andor iXon EMCCD in combination with quantum-dot probes 10–20 nm in diameter to watch motor proteins walk on microtubules.

Not content with just observing, Diez is harnessing the power of motor proteins to perform highly specific manipulations, including stretching single DNA molecules as well as networks of DNA. "It's like body surfing," says Diez. "The microtubules are gliding across the surface of the kinesin motors. It's the force of the motors that moves the DNA attached to the microtubules." These kinds of manipulations are highly effective because they employ the cell's own machinery, he explains. "This is actually a good example, not of where nanotechnology helps us to improve biology, but of where biology has been used to improve nanotechnology."

These manipulations could reveal more about DNA mechanics and DNA–enzyme interactions, and also show the potential of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Aspects of a cell: a living fibroblast imaged by atomic force microscopy (first panel, height image; second panel, deflection image) and simultaneously by epifluorescence (third image), and by phase-contrast microscopy (far-right image).

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biological motors for use in the molecular manufacturing of nanoelectronic circuits.

Biochemists generally have to break open and extract the contents of cells to study reactions of interest, often after having to synchronize the cells' activity to get a high enough concentration of the molecules of interest in the same state. But now, thanks to a handful of pioneering research groups, it is becoming possible to study individual protein, DNA or RNA molecules in live cells, in real time, and to follow their interactions and kinetics with nanometre-scale spatial precision and millisecond time resolution.

One at a time

Chemical biologist Sunney Xie and his team at Harvard University in Cambridge, Massachusetts, have developed methods for observing the expression of individual protein molecules. After initially testing them in bacterial cells, the group is now extending the techniques to mammalian cells. This approach is useful for detecting proteins expressed at low copy number, at just a few copies per gene, such as transcription factors, which would be undetectable using conventional proteomics methods.

The great advantage of the single-molecule method is that individual proteins are detected as they are synthesized in an individual cell. One of Xie's techniques detects the expression of proteins genetically tagged with the fast-maturing yellow fluorescent protein Venus. The appearance of the protein, observed as a series of fluorescent bursts that can be quantified, indicates that transcription and translation are taking place.

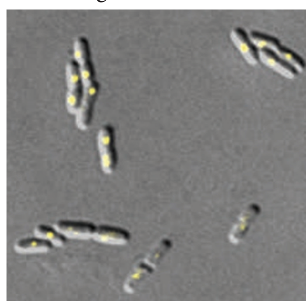
Rapid strobing illumination enables the observation of fast binding and unbinding of fluorescently tagged proteins to immobile components in the cell and of interactions between fluorescent proteins in the cytoplasm. "Like photographic images of a bullet going through an apple, we can detect fluorescent proteins in the cytoplasm. We can play with the pulse width to determine the dynamics and where the proteins are," says Xie.

This technology could be extended to a high-throughput system, according to Xie's postdoc Nir Friedman. "You could put cells on a large chip with many chambers and do these kinds of measurements on a large scale."

The fluorescent protein fusion could be done with a DNA library in order to detect the expression of proteins of unknown function. "Although you need to target specifically, the advantage is that you can look at live cells in real time and with single-molecule sensitivity," Friedman adds.

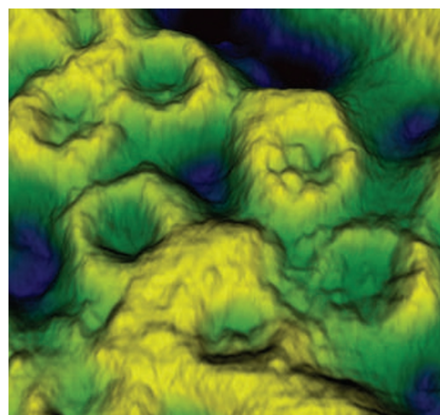
Nanotechnology seems destined to leave a lasting legacy for cell biology with a host of innovative new technologies. With continuing efforts to combine existing technologies in novel ways, and to create new ones, the possibilities for gaining new insights through nanoscale cell manipulation are increasing rapidly. Nanopatterning and nanotopography are techniques that are, as yet, practised by only a handful of specialists, but the equipment and software are fast becoming available commercially. This trend towards the increasing use of nanotechnology is pushing the very boundaries of cell biology. ■

Hayley M. Birch and Julie Clayton are science writers based in Bristol, UK.



J. ELF & G.-W. LI, HARVARD UNIV.

One at a time: single fluorescently labeled proteins binding at one site per *Escherichia coli* chromosome.



B. WINDOFFER, UNIV. MUNSTER/JPK

A 3D image of a nuclear pore complex.

DOWN TO THE LETTER

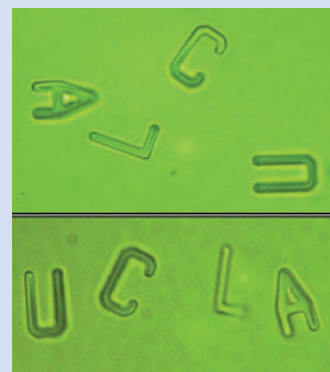
Nanoprobes come in all shapes and sizes. In the latest advance in probe engineering, chemists, physicists and engineers at the University of California, Los Angeles, are pooling their resources to perfect a method for mass-producing novel fluorescent microparticles. The nature of these particles can be so precisely controlled that researchers have been experimenting by creating entire alphabets that can be manipulated with optical tweezers, raising the intriguing possibility of playing nano-scrabble.

These so-called LithoParticles are sculpted by electron-beam lithography, directed by the same computer-aided design (CAD) software used by architects. "E-beam writing is a serial process," says Thomas Mason, who leads the group. "Each letter is written one at a time, so it's not very good for mass production. However, once the mask is made, it can be used over and over again in a special optical-projection printer. We use a mask made by E-beam lithography to expose resist-coated wafers to patterned ultraviolet light. A different projection-printing device — an optical lithography system known as a stepper — is used to mass-produce many particles in parallel." The Ultratech XLS stepper has a lens weighing over 90 kilograms and its own heating and air-conditioning systems to control thermal expansion. The same technology could be used to mass-produce particles with feature sizes as small as 30 nm.

The potential implications for cell biology are huge. Such accuracy of design, coupled with high fidelity on a mass scale, means researchers could soon be supplied with solutions of probes tailored to their specific needs, as neatly demonstrated by Mason's 'alphabet soup'.

Nanoprobes are being increasingly used in the emerging field of bio-microrheology, which examines transport processes within living cells, and in investigating the mechanical properties of cellular components. Nanoparticles introduced by ballistic injection have revealed how the cytoplasm of human umbilical vein endothelial cells undergoes elastic changes in response to growth factors. But the approach could be expanded to investigate the cell's response to all manner of different shapes. "Tracking how differently shaped particles move and rotate inside cells may provide a wealth of information about life cycles and internal cytoplasmic transport in different cell types," says Mason. "You could also use these probes to study how cells respond to various external stimuli. For instance, particles that have many long 'arms' may behave very differently to the compact spheres and quantum dots that are currently available."

UCLA is currently applying to patent their technology and are involved in discussions with commercial partners. Mason is already speculating about building functional nanomachines — including motors, pumps and entire engines — which could be sent to probe even further into the workings of the cell. H.M.B.



J. N. WILKING/T. G. MASON/UCLA

Under the spell: nano-alphabet.

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