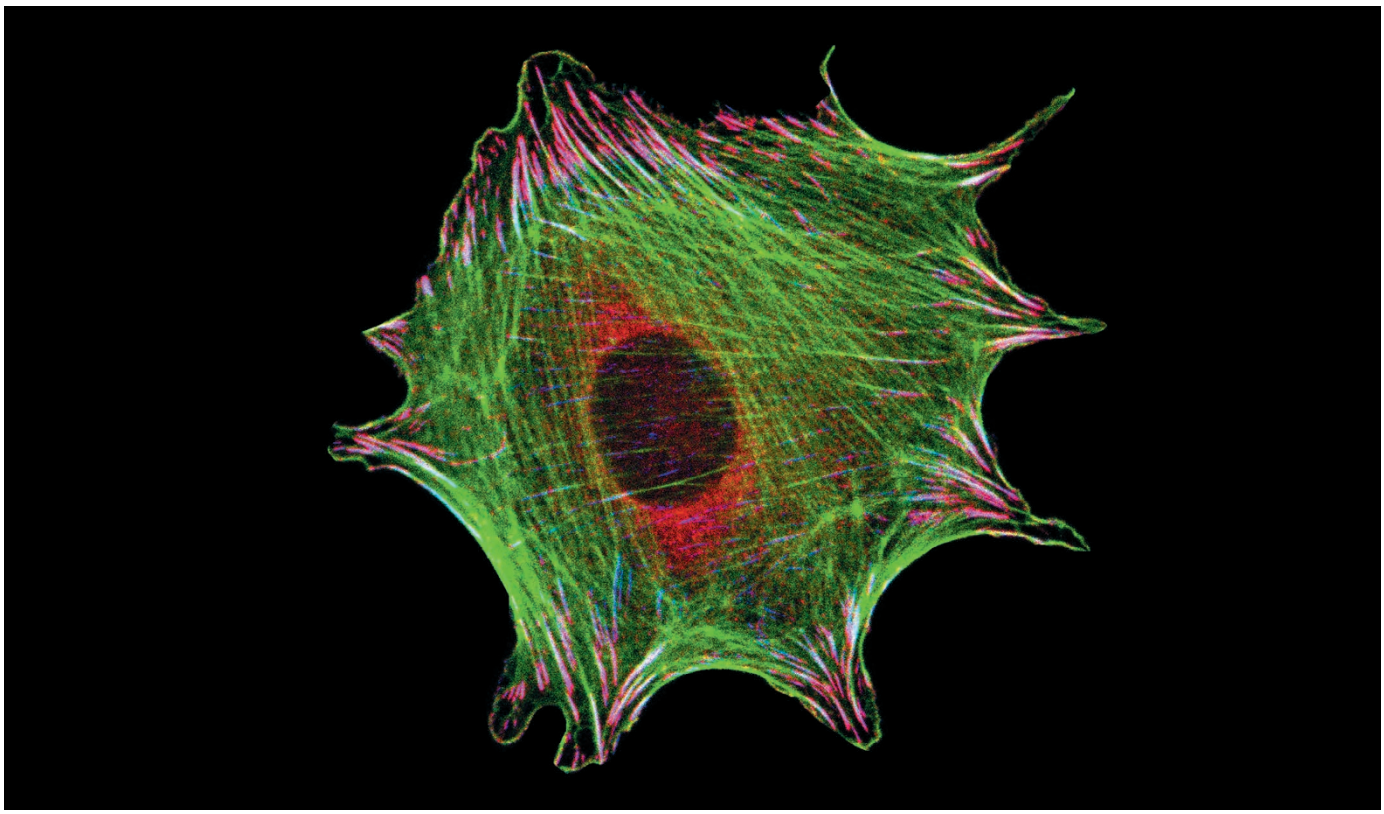
TECHNOLOGY FEATURE

A MEASURE OF MOLECULAR MUSCLE

Innovative tools are revealing the forces that guide cellular processes such as embryonic development and tumour growth.

CARSTEN GRASHOFF



Protein structures in cells experience and create tiny forces that scientists are learning how to map at the micro scale.

BY MICHAEL EISENSTEIN

Under a microscope, cells often appear static — solid elements of biological architecture. Yet in reality, they are dynamic structures, jostling for space in a packed environment. Cells squeeze, stretch, flex and pull on their surroundings and each other, exerting force as they do so. These forces are incredibly small — on the scale of piconewtons, or roughly one-billionth of the weight of a paperclip. But they can have profound biological impact. The shifting forces in a rapidly growing embryo can alter cells' developmental programs, telling them when to stop dividing and begin transforming into brain or bone.

The idea that physical forces affect cellular function was put forth a century ago, by Scottish scientist D'Arcy Thompson in his seminal work *On Growth and Form*[1]. "Cell and tissue, shell and bone, leaf and flower, are so many portions of matter," wrote Thompson, "and it is in obedience to the laws of physics that their principles have been moved, moulded and conformed."

Thompson's largely theoretical work paved the way for numerous experimental studies of biomechanical principles. "Biomechanics is a very old field — people just ignored it for a long time," says Carsten Grashoff, who studies cellular forces at the Max Planck Institute of Biochemistry in Munich, Germany. In part, that's because researchers lacked the technology to measure molecular-scale forces with precision.

Now, scientists have tools that make it possible to microscopically map the forces that skin cells exert as they crawl forward during wound healing, or to program cells to blink on and off as proteins stretch and relax. Hurdles remain — researchers still struggle to tell apart the true effects of cellular forces from random biological noise, and they have yet to work out how these processes play out in the complex environment of a living organism. But by combining 'mechanobiology' tools with other measurements of genetic and biochemical activity, researchers can begin to understand how force is translated into function.

"Life processes aren't just a biochemical signalling pathway," says Beth Pruitt, a mechanical engineer at Stanford University in California. "When you pull on a protein, ▶

you might open or close a binding site that flips a switch to choose which program is going to be run by a cell."

GAINING TRACTION

Cells interact with their environment largely through proteins embedded in their membranes, which represent crucial foci for generating and interpreting cellular forces. Some proteins respond to being 'pushed' by the flow of liquid, as seen in blood vessels, whereas others generate tension-related signals when a cell gets tugged by its neighbour or latches onto other proteins nearby.

A method called traction force microscopy (TFM) took off in the 1990s, and gave the field its first real tool for quantitatively measuring such forces. In 1999, for example, Yu-Li Wang, then at the University of Massachusetts Medical School in Worcester, and Micah Dembo at nearby Boston University, plated connective-tissue cells called fibroblasts on a gelatinous material embedded with fluorescent beads. They then showed that they could use TFM to deduce the forces that those cells generated by measuring the displacement of the beads[2]. "It's like a spring-scale," says Ben Fabry, a biophysicist at Friedrich Alexander University of Erlangen-Nuremberg in Germany, "where you put a weight onto a spring and measure the deformation, and you can determine the force if you know the stiffness of the spring."

TFM has become a standard method for studying both individual cells and tissue-like sheets of interconnected cells. Clare Waterman at the National Heart, Lung and Blood Institute in Bethesda, Maryland, has used TFM to study cell migration, a process mediated in part by the forces that cellular structures known as focal adhesions exert as they anchor themselves onto the surrounding extracellular matrix (ECM).

Waterman's group has developed strategies for increasing the number of beads that can be imaged in a TFM experiment, producing ultra-high-resolution force maps. "We can get 50 markers under each focal adhesion, so we're down to submicrometre resolution," she says. This has enabled her group to reveal how forces generated at focal adhesions trigger the molecular events that coordinate directed cellular movement during processes such as embryonic development[3].

Of course, the multidimensional shifting of beads in response to cellular motion is much more complicated than a one-dimensional spring scale. TFM initially required powerful supercomputers to interpret the data, although modern computational methods have made the technique more accessible. Even so, converting bead-displacement data into force measurements remains challenging, and there are many sources of potential error. "You can have a single cell pulling in opposite directions that makes it look like there's basically no deformation," says Waterman. "And when there's movement of beads well beyond the boundaries of the cell, that can be hard to deal with."

Other groups are extending TFM into three dimensions, in an effort to better mirror biological reality. Fabry and his colleagues, for example, developed a TFM method to track cellular forces in 3D using gels built of collagen, a key protein component of the ECM. His team was able to probe the relationship between the shape of breast cancer cells, the forces that they generate and their speed and direction as they propel themselves through a synthetic 3D 'tissue'—potentially modelling metastatic growth[4].

"Mechanical forces can play as big a role in the eventual shape of a tissue as the genes that are activated."

But his method also ups the analytical and computational challenges. "Collagen is a very awkward material — its behaviour is highly nonlinear, meaning that if you stretch it a little bit it's soft, but if you stretch it a bit more it's suddenly very stiff," he says.

To work around this computational burden, Celeste Nelson, a biomedical engineer at Princeton University in New Jersey, settles for lower-resolution data in her studies of organ development. "We care more about finding the relative differences in the magnitude of force across an entire population of hundreds or thousands of cells," she says. However, force generation is inherently dynamic; by collecting these 3D data at multiple time-points, she says, "the computational demand just explodes".

ON PINS AND NEEDLES

As a simpler option, some researchers use small, precisely designed chips moulded from various polymers, which provide a direct readout of cellular forces. One widely used design, developed by bioengineer Christopher Chen of Boston University, Massachusetts, and his colleagues, consists of an elastic material called PDMS that displays an array of flexible pillars, like the bristles on a toothbrush. These 'micropillars' are topped with ECM proteins that allow cells to form attachments[5]. "They basically act like mini springs," says Jianping Fu, a former postdoc of Chen's who now uses similar devices to study human stem cells at the University of Michigan in Ann Arbor. "By measuring the deflection, people can identify and measure the forces that cells exert at individual pillars."

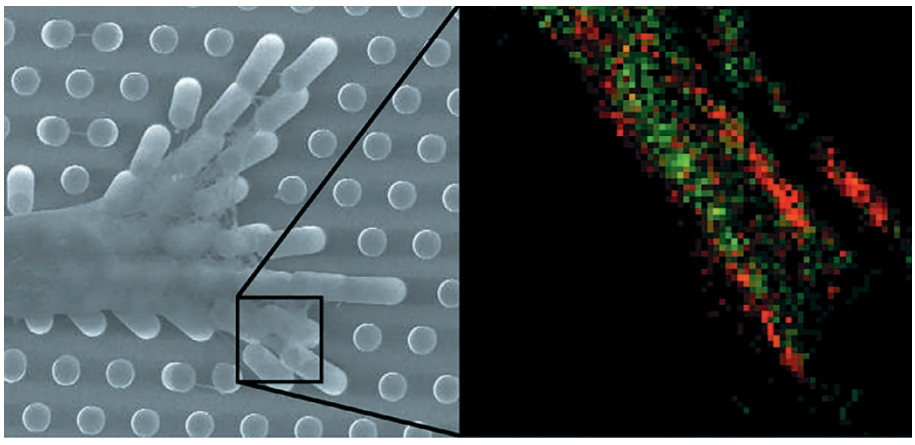
Micropillar-array data are easier to interpret than the results of TFM experiments, and they require less computational analysis. And the devices themselves are reasonably straightforward to manufacture. They are also compatible with fluorescence microscopy, which facilitates molecular-scale investigation of the events that produce cellular forces. However, these arrays also impose a specific — and unnatural — pattern of interaction between cells and their substrates, governed by the distribution and size of the pillars, which could deviate from how cells behave in living organisms.

Researchers can also customize the culture surface by manipulating the design of the micropillar array. Shorter, thicker pillars are more rigid and unyielding, whereas taller, slender pillars are flexible and more responsive to force. Such changes in the rigidity of the pillar surface can trigger considerable reorganization of a cell's cytoskeleton — the network of proteins that form the cell's physical infrastructure and help it to transmit and respond to force. This, in turn, can influence cellular proliferation, movement and maturation.

Fu, for instance, has found a relationship between surface rigidity and adult stem-cell differentiation. "With stump-like pillars that are hard to bend, they become bone cells, but when you seed them on taller pillars, they have a greater tendency to become fat cells." By precisely tuning the design, Fu's team was even able to develop a culture system that strongly favours the development of human embryonic stem cells into functional spinal motor neurons[6].

TINY TUGS

Other researchers are measuring the forces applied by proteins or protein complexes using molecular sensors that generate a fluorescent signal in response to small-scale changes in tension. Such sensors generally are based on Förster resonance energy transfer (FRET), a



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Deflected 'micropillars' in experimental arrays can be measured and compared with cell protein scaffolds.

NIKOLCE GJOREVSKI

phenomenon in which one fluorescent molecule, or fluorophore, excites another — but only when they are in close physical proximity.

As a postdoc in the lab of biomedical engineer Martin Schwartz at the University of Virginia in Charlottesville, Grashoff worked with colleagues to develop one of the first FRET-based tension sensors, which they described in a 2010 paper[7]. "We had this idea that we would use this very elastic protein that you find in spider silk to link the two fluorophores to each other," Grashoff says. "When there is mechanical tension, it would elongate, and you could measure the decreased FRET signal." At rest, the silk protein forms a compact coil, but a gentle tug can stretch out the spring and uncouple two flurophores.

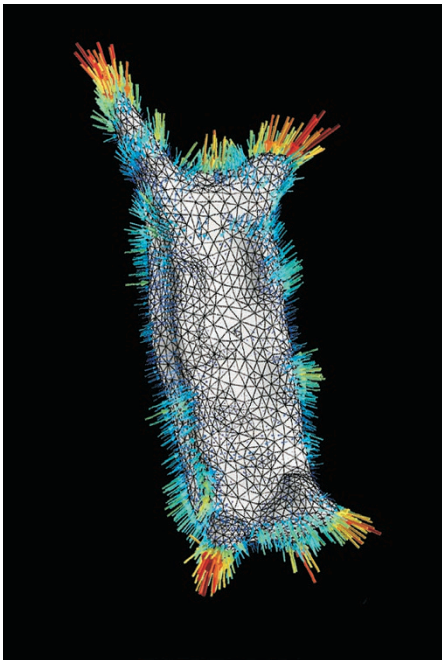
As proof of concept, Grashoff and Schwartz incorporated their sensor into a protein called vinculin, a component of focal-adhesion complexes that form a bridge between the ECM and a cell's cytoskeleton. When they expressed the sensor protein in cells, they observed that vinculin experiences piconewton-scale forces that change as focal adhesions assemble and disassemble during cell migration[7].

In principle, FRET sensors can be inserted into a diverse range of proteins to obtain measurements otherwise difficult to collect. For example, there are few tools for quantifying how cells push and pull on each other in a tissue. Stanford University chemical engineer Alexander Dunn and his colleagues were able to measure these forces by integrating Grashoff and Schwartz's FRET sensor into E-cadherin, a protein that couples cells together[8].

And sensors can be built around other linker proteins, as well, enabling researchers to fine-tune a sensor's sensitivity. Grashoff notes that his FRET-sensor collection can selectively respond to forces of 1–12 piconewtons. But there's still room for improvement, he adds, because intracellular proteins can experience forces as high as 20–30 piconewtons.

But the design and validation of such sensors is labour-intensive, and sensors can 'go dark' for reasons other than force detection — such as degradation. FRET signals can also be challenging to interpret, requiring careful measurement to eliminate false positives. And there are consequences from inserting a bulky sensor into proteins whose function is strongly structure-dependent. "You cannot fully predict how well it will work," says Grashoff. "It may not be a problem that you've disturbed the protein, but you have to know how great that disturbance is."

Other groups are measuring extracellular forces using sensors that need not be shoehorned into a protein. Biophysicist Khalid Salaita's group at Emory University in Atlanta, Georgia, has developed several such probes, in which one end is anchored to a solid surface, such as a glass slide, and the other displays a biomolecule that binds to a cell-surface protein of interest[9]. In between, Salaita places various linkers that are responsive to forces of different magnitudes. "Polyethylene glycol polymers are like wet spaghetti, where you have this random coil that gets stretched, whereas DNA has this fixed secondary structure," says Salaita.



A map of traction forces exerted by migrating cells.

His group also uses linkers derived from a protein called titin, which produces elastic recoil in muscle. "It's a naturally evolved spring that can withstand greater forces," says Salaita. These titin-based sensors were strong enough to measure the powerful pull that cells exert on their environment through integrin proteins[10], a component of focal adhesions that can generate tens of piconewtons of force — enough to tear apart weaker DNA duplexes and polymer-based sensors. "The integrin is basically a brute-force grappling hook that anchors cells and applies extensive forces," says Salaita.

FORCES FOR GOOD

That scientists can measure intracellular forces is itself remarkable. The resulting insights might yield valuable clinical dividends. Salaita thinks that assays for measuring forces at the single-cell level could help scientists to identify safer drugs that interfere directly with physical mechanisms of tumour progression. "The migration and the invasion of the cancer cell is what is most deadly, and if you can shut down that mechanical process but make the drug non-cytotoxic, that's a much more precise tool," he says.

However, many biological questions need to be explored at the tissue or organ scale. "You can't predict much about a tissue from isolated cells," says Nelson. "The connections of individual cells seem to be necessary for the generation and transmission of force in a tissue."

In many of her experiments, Nelson uses engineered epithelial tissues that provide a controlled, reproducible model for studying the forces involved in organ formation. Other groups use stem cells to generate specialized tissues; for example, Pruitt uses stem-cell-derived cardiomyocytes to study the mechanobiological effects of heart disease. "We have this confluence of tools that makes it possible right now to create cardiomyocytes from human cells and apply and measure force, displacement and stretch in single cells and microtissues," she says.

Nelson is excited that scientists are finally able to explore the implications of Thompson's century-old hypotheses. "I think the field as a whole is revealing that mechanical forces can play as big a role in the eventual shape of a tissue that develops as the genes that are activated during the process — if not bigger," she says.

Still, more tools are needed. Most force-measurement experiments remain time-consuming, which limits their usefulness for applications, such as drug screening, that require parallel analysis of large numbers of cells. Fabry's group is developing ways to automate and accelerate TFM experiments. "We want to measure the response of hundreds or thousands of cells in a 3D tissue at the same time," he says.

Measuring cellular forces in living organisms is also a significant challenge. FRET sensors offer one solution, and mechanical engineer Otger Campàs at the University of California, Santa Barbara, and his colleagues recently devised another. His group injects living organisms with fluorescently labelled oil droplets, which are decorated with proteins that can bind to cell surfaces[11]. By documenting how these droplets deform in the spaces between cells, they can computationally derive the 3D forces those cells are exerting on the droplets.

Perhaps most fundamentally, there is a need for experimental techniques that allow scientists to manipulate force-responsive molecules more precisely. The ability to inactivate individual focal adhesions, for instance, similarly to how geneticists knock out individual genes, could reveal how the timing and spatial distribution of molecular forces alters their effect on cells, Nelson says. "That would allow us to directly answer many questions that we're kind of dancing around right now." ■

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