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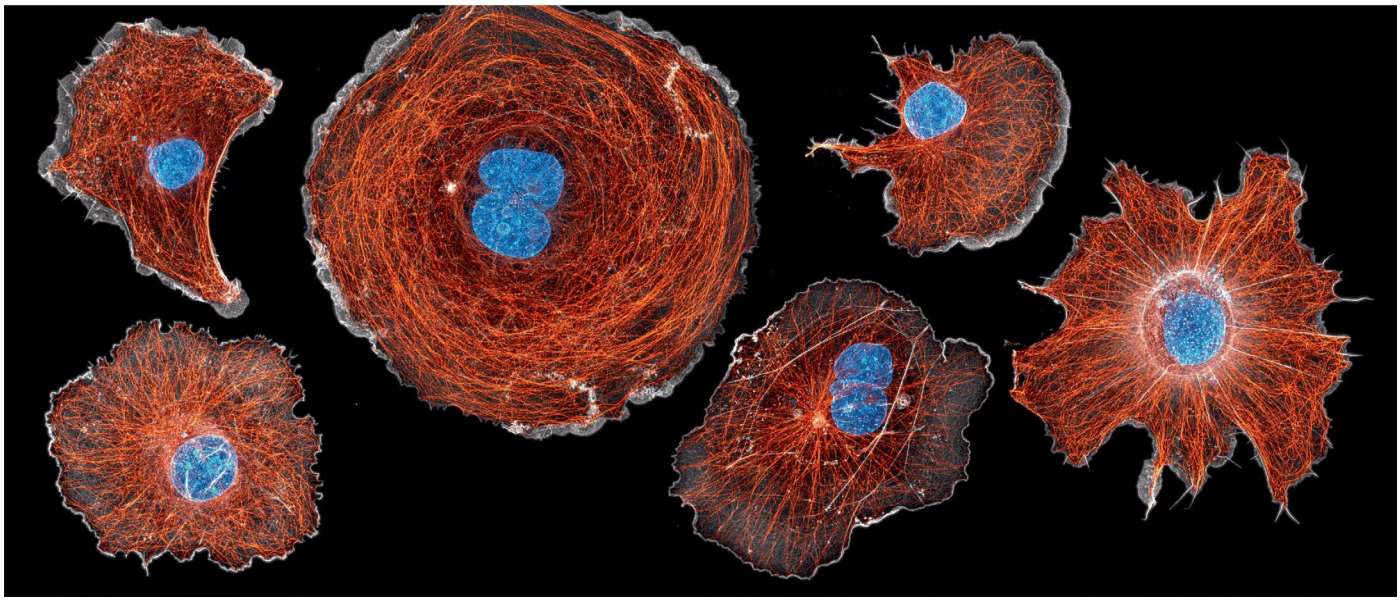

Seeing the cytoskeleton is observing mechanobiology. Here, in monkey kidney-derived fibroblasts, actin is gray, microtubules are orange and DNA is blue.

Actin in action

To gain insight into cell function, researchers are tracking the cytoskeleton and its parts, such as actin. They combine methods, find new trackers and validate them.

By Vivien Marx

Among the ways to assess the cell's mechanodynamics are methods to characterize actin filaments and other cytoskeleton components. Some labs apply labels and imaging techniques; others reconstitute aspects of the cell's mechanobiological to-and-fro in vitro or model them computationally.

The cytoskeleton is bit like a railroad track network. It's made of actin filaments and microtubules that molecular motors such as myosins and kinesins travel on.

In "Lighting up the Mechanome," Vanderbilt University researcher Matt Lang notes that 'omics approaches in genomics and proteomics are "systems-level tools." But for the "mechanome," which describes the role of force, mechanics and machinery in biology, such an 'omics perspective is lacking. Probing "nature's machinery" is about gaining a quantitative understanding of molecular and cellular machinery and measuring the forces and strength underlying the "mechanome." Among the tool and assay advances are force microscopy for exploring how structures react to controlled loads or optical traps for probing molecular interactions of biological motors or the interactions of receptors and ligands.

The cellular "mechanome" concept conjoins key aspects that matter to her lab, says Nikki Reinemann from the University of Mississippi, who completed her PhD research in the Lang lab. Characterizing the cytoskeleton takes a systems-level approach, in her view. The cell works with a range of building blocks with a variety of properties, she says. Microtubules and actin filaments have different levels of stiffness; motor proteins have varying motility and facilitate different types cellular processes. Filament crosslinkers can be pliable in different ways and have various types of propensities to build hierarchical geometries. "These disparate Lego pieces must work together and communicate across large scales in order for robust cellular processes, such as motility and division, to take place," she says. Additionally, there's a layer of emergent cytoskeletal mechanics, which refers to collective behaviors and interactions of the many components that are more than the proverbial sum of their parts. Together, this is a "fascinating design challenge for the next generation of mechanobiologists and biophysicists."

Christophe Leterrier is a Centre National de la Recherche Scientifique (CNRS) researcher at the Institute of NeuroPhysiopathology, which is affiliated with CNRS and Aix Marseille University. Leterrier trained in physics and chemistry and became a neurobiologist because the cytoskeleton is everything that drew him to biology: "a fascination for intricate and dynamic assemblies that ultimately control the cell growth, shape, movements and reaction to its environment." The cytoskeleton is all about making myriad different structures with a limited set of bricks – actin and tubulin – "which can only appeal to a Lego-fan engineer." It's more tangible and amenable

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to structural thinking than, say, signaling pathways. For structure-to-function science, the cytoskeleton is the perfect substrate. His lab is devoted to determining the spatial and dynamic arrangement of the cytoskeleton at the best possible resolution to shed light on its function in a testable way. Leterrier sees two important tool categories: one helps with visualization and the other with perturbation. In the coming years, capturing the three-dimensional arrangement of molecules in cells will take all the types of microscopy: super-resolution, optical and electron microscopy. Beyond hardware, he says, are innovative probes and new approaches in computational image analysis powered by deep-learning that let researchers move beyond the compromises intrinsic to microscopy. "In this sense, I feel like the cytoskeleton is really the nexus of interdisciplinarity in today's cell biology," he says.

In her lab, Nikki Reinemann (top left), graduate student Omayma Al Azzam and the rest of the team combine fluorescence microscopy with high-resolution optical trapping, in which a laser beam is used to measure the forces that cytoskeletal proteins generate.

Seeing the dynamic

In her lab, Reinemann and her team combine fluorescence microscopy with high-resolution optical trapping, in which a laser beam is used to measure the forces that cytoskeletal proteins generate. Hers is a connectedness approach: some projects aim to assess and probe motor proteins on a single-molecule level; others focus on physical properties of the cytoskeleton's building blocks; and others address the properties of engineered cytoskeletal assemblies. She and her team have built 'nanocells' made of ensembles of reconstituted cytoskeletal proteins and use them to study the mechanics of the cytoskeleton with high-resolution optical tweezers[1]. Rather than an in vitro approach focused on one motor protein–filament interaction with a single filament and individual motor proteins, an ensemble-based approach more closely reflects the native cellular environment.

Johns Hopkins University researcher Douglas Robinson and his team, along with others, including mathematicians, are building computational models of cell mechanics to explore factors that shape cell deformations during processes such as cancer growth and progression[2]. Cancer cells and healthy cells are mechanoresponsive, and external mechanical forces shape their gene expression[3]. The scientists have found that elements of the cell's "contractility machinery," such as actin and myosin, associate with one another in the cytoplasm. It's possible, says Robinson, that these "contractility kits" are preassembled to enable a rapid response to mechanical stress or a signaling cue. To computationally model the way myosin and actin interact in contractility kits, the scientists build on experimental observations and measurements made with interaction genetics, proteomics, coimmunoprecipitation techniques and in vivo imaging – in particular, fluorescence cross-correlation spectroscopy, says Robinson. The team is now working to computationally reconstitute these contractility kits and integrate the cell's molecular events, signaling and mechanoreactions in a model. "This requires a very interdisciplinary approach that extends from molecular to cellular scale levels combined with ongoing mathematical model development," he says.

A different kind of model is emerging in an European Union project called the European Synthetic Cell Initiative, in which researchers are building a simple but complete cell from scratch. The teams look at how the molecules of life are organized in time and space to enable basic functions like growth, information transfer and division, says Delft University of Technology researcher Marileen Dogterom, who is part of this initiative. "For mechanobiology, the combination of labels and sensors is very powerful," says Dogterom. Labels are a way to study organizational changes, which are "very profound for the cytoskeletal proteins," and their use can pinpoint where the cell's main mechanical responses take place. Insight into the chronology of changes can come from dynamic and high-resolution imaging techniques. Molecular sensors are useful for extracting details of ongoing processes, such as to measuring forces with force sensors, says Dogterom. "Being able to combine chronology, high spatial resolution and functional details will really move the field forward," she says. It intrigues her to get at mechanism by controlling control processes in systems with adjustable knobs. Optogenetic tools, she says, offer such a knob, "where you turn some interactions on or off where and when you choose."

The actin-probe toolbox has sensors and probes of many types. For instance, Salk Institute researcher Uri Manor and his team

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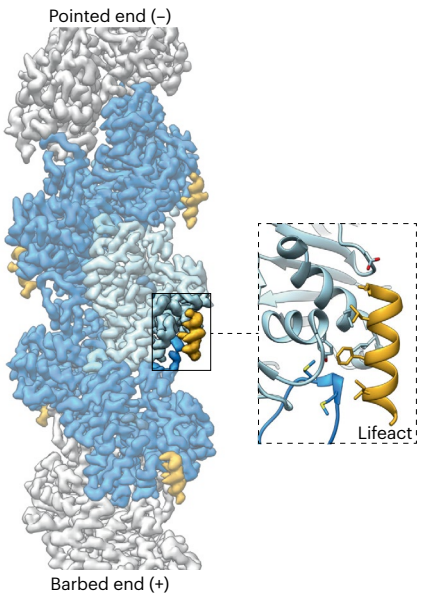


Lifeact is used to label actin in live cell fluorescence microscopy. The Raunser lab used cryo-EM to determine, at 3.5 Å resolution, the structure of the Lifeact–-filamentous actin complex, shown here bound to actin (PDB 7AD9).

developed actin chromobodies[4], which are fluorescently tagged nanobodies linked to actin. These probes can, for example, anchor themselves in the membrane of an organelle such as the mitochondrion, such that labs can assess actin dynamics at that location, he says. The team also developed an actin nanobody tethered to the endoplasmic reticulum. The endoplasmic reticulum membrane is contiguous with the nuclear envelope, and he saw "interesting patterns of actin on the nuclear envelope." Perhaps a mechanical structure is transducing mechanical forces to the nucleus. "Overall, our understanding of how cytoskeletal mechanical forces affect organelles is very poor, which to me means it is very exciting to see so much progress in new probes and tools for studying these subcellular structures," says Manor.

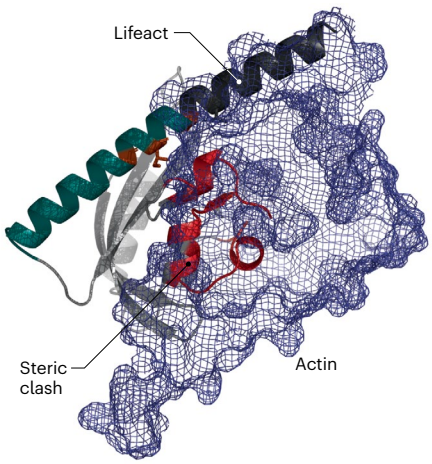
Lives of Lifeact

Actin is the most abundant cytoskeletal protein in eukaryotic cells. "The cell is full of it," says Michael Sixt, a researcher at the Institute of Science and Technology Austria. Actin is involved, for example, in the body's muscle contractions and helps cells move and change shape. Lifeact is a widely used imaging probe for tracking actin[6]. It's a peptide – 17 amino acids short – fused to GFP that stains filamentous actin in eukaryotic cells and tissues. It's been used in around 7,000 studies, says Roland Wedlich-Söldner, a researcher at the University of Münster's Institute of Cell Dynamics and Imaging. He and his team co-developed Lifeact while he was a junior group leader at Max Planck Institute (MPI) for Biochemistry, along with Sixt and his team, who was then his MPI neighbor and a junior group leader, and Zena Werb from University of California San Francisco, who was a visiting researcher at the MPI.

A newer probe is LILAC, an optogenetic variant of Lifeact developed by Ronald Rock and his team at the University of Chicago's Institute for Biophysical Dynamics, along with colleagues at Purdue University[7]. LILAC opens the door to a number of imaging modes that take advantage of photoswitching, says Rock. Because the label moves from the cytosol to the actin filaments, it's possible to do before-versus-after image processing to improve the image contrast.

Paper first author Kourtney Kroll, a graduate student in his lab, hit on that idea on her own, he says. "I love it when a student brings me something creative like that, something that knocks your socks off." LILAC uses a domain from a light-sensitive protein in oat called phototropin1 that is attached to Lifeact. The domain – LOV2, which stands for the second light-sensitive light–oxygen–voltage domain – makes the probe photoswitchable. In reaction to blue light, a conformational change leads LILAC to bind actin. Without light stimulation, LILAC is unbound; it disconnects as a result of steric hindrance between the LOV2 and actin.

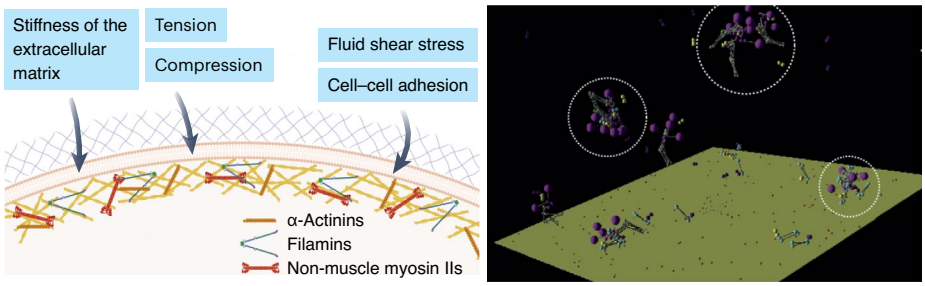
One of the paper's reviewers, says Rock, pointed out optical lock-in detection (OLID), a fluorescence imaging technique in which a probe can be switched on and off over time to highlight image pixels that follow that switching pattern. This lock-in detection scheme can be quite sensitive, he says. "We can do that with LILAC as well, using the switching on and off of actin." One reason the team developed LILAC was to address potential issues some labs have seen with Lifeact, he says. Effects include altered cell shape in stem cells and sterility in fruit flies. When Lifeact binds F-actin, it can stabilize those filaments and affect actin dynamics, says Rock. "This can be a problem unless you're careful to limit the amount of Lifeact in your experiment," he says. Others, too have pointed out concentration-dependent effects[8]. With a photoswitchable label, says Rock, the perturbation is limited to the brief period, when a lab is looking at actin. "The rest of the time, the cell can go on its merry way," he says.



LILAC is a photoswitchable label for actin. Researchers at the University of Chicago led development of this optogenetic variant of the actin label Lifeact, shown here bound to actin (PDB 7AD9). Under blue light it binds to actin. Without light stimulation, LILAC is unbound as a result of a steric clash.

Developers' view

Wedlich-Söldner and Sixt, who were interviewed jointly, both saw a need for a uniform way to label actin. Plant labs used different approaches from yeast labs, and labs working with mammalian systems used yet others. Before Lifeact, labs could, for instance, study a mouse in which globular actin was fused to GFP and expressed, but the actin signal was either absent or quite dim, says Sixt. In the Lifeact actin reporter mouse, they believe the animal tolerates high levels of Lifeact expression. Almost all cell types express actin, says Sixt, and a "nice signal" is generated, he says. The mouse has no discernible phenotype. Says Wedlich-Söldner, he has been excited to see cryo-electron microscopy (cryo-EM) images of Lifeact bound to filamentous F-actin[5]. It has shown decorated actin filaments in which almost every subunit of actin has Lifeact bound to it. Even at that saturation, it does not alter actin structure, he says. Lifeact is currently 17 amino acids long, with a shorter version in the works. Early tests indicate it works just as well. With Lifeact, "we tried to replicate some of the defects that people have published," says Wedlich-Söldner, but even at a concentration two orders of magnitude higher than detection levels, "we still don't see them." To him, the defects appear connected to Lifeact



Left, cancer cells are mechanoresponsive. External mechanical forces shape gene expression and cancer progression. Right, computational models help to capture details of cell mechanics and interrogate drivers of cell shape changes or interactions between a cell and its substrate.

overexpression or, at least in part, GFP overexpression. "If you overexpress – massively – Lifeact, you absolutely misorganize actin, and you kill every cell," he says. He recommends labs tune the Lifeact concentration to the minimum needed for observation. Says Sixt, labs should know their experimental system to assess it for possible artifacts and use the right controls. After developing the probe, "we gave it to over 400 groups before it was published," says Wedlich-Söldner. In their distribution note, they described potential problems. "I didn't hide that, and I think that helped a lot to get it accepted," he says.

They both like LILAC. In its "dark phase," the marker is "non-functional and non-disturbing" to the cell, says Wedlich-Söldner. "It's also a really cool idea," he says, to bring together two short peptides with complex conformations and use that proximity to control interaction with their respective ligands. Given how densely covered actin's surface is, he was surprised one can place a second peptide right next to Lifeact and achieve this control. LILAC would be the right choice for labeling actin in longer-term experiments, such as ones with organoids cultured for weeks or even months, he says. To follow differentiation over time, LILAC could be turned on when needed for imaging sessions.

Says Rock, "To be clear, we're big fans of Lifeact as well." Seeing actin in live cells matters, and he agrees that LILAC will work well when experimenters want to keep a cell population healthy over a long time span and use various imaging modes.

Visualization and perturbation techniques come across as separate categories, says Leterrier, but Lifeact highlights that "this is not a clear-cut divide." As in quantum physics, the introduction of probes for imaging "always has a perturbative effect on the system, and so does the observation itself," he says. One has to consider phototoxicity or probe size when using super-resolution microscopy or electron microscopy. "So we're always walking the fine line of performing the best and most resolutive imaging without perturbing the system too much," he says.

Proper characterization and controls matter to tell experimentalists where they stand, to gauge whether the data they obtain are meaningful, says Leterrier. LILAC's development indicates that it is promising to work on spatiotemporal control of probes and perturbators, "so that you have them active only when and where it's needed, avoiding long-term or cell-wide side-effects." Along these lines, he can think of two tools that would be helpful for cytoskeleton research, he says. One would be a small-molecule fluorescent probe for microtubules, similar to phalloidin for actin, for simple and robust labeling of microtubules in fixed cells with the highest possible density for super-resolution microscopy. The other is an optically controlled actin perturbation tool for disassembling actin with spatiotemporal control, one similar to the optogenetic tool opto-katanin for microtubules, he says.

What your reporter does

His rule of thumb, says Robinson, is to use two or more types of reporter that look at the system from different angles. "Especially for actin, which is a highly allosteric polymer, I have never really trusted any single probe," he says. Observer effects can result if a lab places all its trust in just one probe. "I think it is important to test assumptions in each system," he says. "We know of many other examples where 'unappreciated assumptions' may have bold consequences."

Commenting on Lifeact and more generally, Reinemann says with tool use it's important to stay aware of experimental limits and to interpret results within that context. Each established tool in mechanobiology has its benefits and downfalls. That's another reason to foster interdisciplinary collaboration when studying fundamental mechanobiological questions.

He sees uses for a probe that could bring cargo to actin, says Sixt. "Subcellular targeting of effectors is definitely interesting." In recent work on events in a swelling lymph node[9], he and his colleagues used two transcription factors – YAP and TAZ – to detect tension on the cytoskeleton. Under tension, these transcription factors move from the cytosol to the nucleus, which makes them an indirect readout of intracellular forces. He would like to see a Lifeact probe that reports on the tension acting on the cytoskeleton "so that you really know what kind of forces is the cell producing and experiencing." In relation to actin imaging and analysis, he would like to track turnover in a living cell, says Wedlich-Söldner. "Because Lifeact is so weakly binding, it basically goes on and off on a millisecond timescale." As an actin filament grows, Lifeact can help to track that, "but you cannot really look at the turnover" and assess the composition of the cell's pool of actin monomers at any given time. Subunits of actin are plentiful in the cytosol, but they are mainly attached to proteins. "There's a usable pool," he says, of unknown size. "There's a clear, big gap still in the field that Lifeact cannot address." Says Sixt, the small amount of actin in the nucleus would also be interesting to image, but its low levels make that a tough task. "You have to crank up the volume so much that everything else shines," he says. Says Wedlich-Söldner, one general pitfall with actin labeling is that the binding event can concentrate actin at a given location.

"Lifeact is really awesome, and not least because it is open source," says Manor. He has developed nanobody-based actin probes, but the nanobodies must be licensed through ChromoTek. His lab has also made and validated Lifeact versions of their organelle-targeted actin probes and put them on Addgene. The issue with any and all actin probes, including Lifeact, says Manor, is that they will have differential binding and thus differential effects on whichever structures they bind to. "Even phalloidin, perhaps the smallest actin-binding probe we use in the lab, will have differential localization depending on which fluorescent dye it is conjugated to," says Manor. Thus, it's important validate the efficacy of one's probe before investing too much time and effort in making cells or animals or drawing conclusions. He and his team navigated issues with nanobodies

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by using an ultralow-expression promoter, "but that means our signal was super weak," he says. "Always some kind of compromise somewhere."

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