FEATURES

# SEPARATION

## Phase separation, an idea about how cells organize their contents and functions into dropletlike compartments, has divided biologists

*By* **Mitch Leslie**

For 7 years as president of the Howard Hughes Medical Institute, Robert Tjian helped steer hundreds of millions of dollars to scientists probing provocative ideas that might transform biology and biomedicine. So the biochemist was intrigued a couple of years ago when his graduate student David McSwiggen uncovered data likely to fuel excitement about a process called phase separation, already one of the hottest concepts in cell biology.

Phase separation advocates hold that proteins and other molecules self-organize into denser structures inside cells, like oil drops forming in water. That spontaneous sorting, proponents assert, serves as a previously unrecognized mechanism for arranging the cell's contents and mustering the molecules necessary to trigger key cellular events. McSwiggen had found hints that phase separation helps herpesviruses replicate inside infected cells, adding to claims that the process plays a role in functions as diverse as switching on genes, anchoring the cytoskeleton, and repairing damaged DNA. "It's pretty clear this process is at play throughout the cell," says biophysicist Clifford Brangwynne of Princeton University.

The pharmaceutical industry is as excited as some academic researchers, given studies linking phase separation to cancer, amyotrophic lateral sclerosis (ALS), diabetes, and other diseases. Dewpoint Therapeutics, a startup pursuing medical treatments targeting cellular droplets, recently signed development deals worth more than $400 million with pharma giants Merck and Bayer. And three other companies looking to exploit the process opened their doors late last year. Reflecting that enthusiasm, *Science* picked phase separation as a runner-up in its 2018 Breakthrough of the Year issue.

Tjian says he was agnostic at first about the importance of the process. But after McSwiggen's findings inspired him and colleagues to look more closely at the range of claims, the researchers began to have doubts. Tjian and a camp of similarly skeptical biologists now argue that the evidence that liquidlike condensates arise naturally in cells is largely qualitative and obtained with techniques that yield equivocal results—in short, they believe much of the research is shoddy.

Moreover, the contention that those intracellular droplets perform important roles "has gone from hypothetical to established dogma with no data," says Tjian, who stepped down as president of Howard Hughes in 2016 and now co-directs a lab at the University of California (UC), Berkeley. "That to me is so perverse and destructive to the scientific discovery process."

Although proponents of phase separation bridle at some of those criticisms, many scientists agree that the research requires a jolt of rigor. "I don't think the whole field is bunk," says biophysicist Stephanie Weber of McGill University. "But we do need to be more careful" in identifying instances of phase separation in cells and ascribing functions to them.

The process may be less important than many scientists now assert, adds quantitative cell biologist Amy Gladfelter of the University of North Carolina, Chapel Hill. Some researchers, she says, have tried to make it "the answer to everything."

**PHASE SEPARATION COULD ANSWER** a fundamental question that has nagged biologists for more than 100 years: How do cells arrange their contents so that the molecules necessary to carry out a particular job are in the right place at the right time? One obvious way is with internal membranes, such as those fencing off the Golgi bodies and mitochondria. Yet many other well-known cellular structures, including the nucleolus—an organelle within the nucleus—and the RNA-processing Cajal bodies, lack membranes.

Phase separation is an appealing answer. Many proteins sport sticky patches that attract other proteins of the same or a different type. Test tube studies have shown that under certain conditions, such as when protein concentration climbs above a certain level, the molecules may begin to huddle, forming dropletlike condensates. Researchers understand the mechanics best for proteins, but nucleic acids such as RNA could also aggregate with proteins. If the process happens in the cell, it could generate and maintain organelles and permit unique functions. "It's a principle that could explain how many things in the cell and nucleus are organized," says biophysicist Mustafa Mir of the University of Pennsylvania, who as a postdoc once worked with Tjian.

Although biologists mooted a role for intracellular droplets as far back as the 1890s, evidence that they are vital began to coalesce a little over 10 years ago. Brangwynne, then a postdoc at the Max Planck Institute of Molecular Cell Biology and Genetics, was tracing P granules, flecks of protein and RNA that, in nematode embryos, mark the cells that go on to produce sperm and eggs. To

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observe the granules' movements, Brangwynne squeezed worm gonads that harbor the structures between two microscope cover slips. Under pressure, P granules responded not like solids but like liquids, flowing along the surface of the nucleus and dripping off, he and colleagues reported in *Science* in 2009. The granules' watery behavior "was mind-blowing. It was so different than anything in cells," says Weber, a former postdoc of Brangwynne's.

In 2012, Brangwynne and colleagues saw similar fluid features in the nucleolus, a dense mix of proteins, RNA, and DNA that manufactures ribosomes, the cell's protein factories. The same year, biophysicist Michael Rosen of the University of Texas Southwestern Medical Center and colleagues showed that three proteins that collaborate to organize part of the cytoskeleton form liquid droplets in a test tube solution. They found that the process speeds the assembly of one type of skeletal fiber in vitro—and might do the same in the cell. Scientists have since reported dozens of examples of cellular structures that are round, prone to fuse, and tend to bead on or flow across surfaces—hallmarks of droplets formed by phase separation (see graphic, p. 338).

To confirm that a molecular gathering in a cell is a liquid and not something more solid, scientists often deploy a technique called fluorescence recovery after photobleaching (FRAP). Using a cell that contains fluorescent proteins, researchers zap the region in question with a laser to darken the molecules and then trace how long the fluorescence takes to diffuse back in from other parts of the cell. A liquid, which the fluorescent proteins easily penetrate, should light up more quickly than a solid. Another test involves applying 1,6-hexanediol, a compound that fractures some of the molecular interactions that hold droplets together, to determine whether the structure dissolves.

Rosen notes that three papers published last year in *Cell* offer some of the strongest evidence for phase separation in cells. One, from Brangwynne's lab, showed a particular protein had to reach a threshold concentration in cells to allow formation of stress granules—organelles that pop up during hard times and have been attributed to phase separation. The other two studies also identified threshold conditions for phase separation. Because a threshold is an attribute of the process, the studies provide "good but not perfect data that these structures are going through phase separation," Rosen says.

Many researchers are now convinced that phase separation explains many aspects of cell organization and function. Several research groups have reported that the mechanism helps convene the hundreds of proteins that carry out transcription, the process of reading DNA to produce the RNA instructions for making proteins. Similar molecular corralling may underlie functions including memory in fruit flies, immune cells' responses to pathogens, DNA silencing, transmission of nerve impulses across synapses, and reproduction of SARS-CoV-2, the pandemic coronavirus.

Conversely, phase separation may cause disease when it goes awry. In 2018, for example, biophysicist Tanja Mittag of St. Jude Children's Research Hospital and colleagues revealed that mutations that promote several kinds of tumors disrupt the ability of the protein SPOP, which helps eliminate proteins that spur growth of cancer cells, to form droplets in test tube solutions. The researchers proposed that phase separation is key to SPOP's cleanup function in cells, and thwarting it allows cancer-promoting proteins to accumulate.

Faulty phase separation could also spur damage by aiding the formation of the toxic intracellular inclusions, or protein globs, that amass in neurodegenerative illnesses such as ALS, Alzheimer's disease, and Parkinson's disease. For example, in some ALS patients the protein FUS is mutated and forms inclusions in their neurons. In the test tube, the mutated protein condenses into droplets that then morph into furry knots of fibers resembling the inclusions. In 2018, biochemist Dorothee Dormann of the Ludwig Maximilian University of Munich and colleagues discovered a possible reason: The mutated version of FUS shrugs off a protein bodyguard that prevents the normal variety from undergoing phase separation and clumping in the test tube.

**YET THAT SATISFYING PICTURE** may be growing murky as more researchers have raised doubts about phase separation. In 2019, for instance, scientists organized a debate at Wiston House, a posh 16th century manor south of London, in part to mull whether the process helped control gene activity. About 30 participants hashed over the evidence that the process occurs in cells with

the help of "free-flowing champagne," recalls Mir, one of the presenters. The group's conclusion, he says, was that the support for many putative cases of phase separation in cells is shaky.

Tjian, who was not at the meeting, came around to a similar conclusion because of new data from McSwiggen. McSwiggen's early evidence showed that in herpesvirus-infected cells, the replication compartments—clusters of protein and DNA that help produce new copies of the pathogen—are round and merge with each other, suggesting they result from phase separation.

After tracking individual proteins within cells, though, McSwiggen and colleagues determined the molecules diffuse just as fast through the compartments as through the rest of the nucleus. In a true condensate, molecular crowding should have hindered diffusion. Other researchers found the negative evidence compelling when it was published later in 2019, soon after the Wiston House debate. The study is "a really important cautionary tale," Weber says.

The results spurred Tjian, McSwiggen, Mir, and Xavier Darzacq, a cell biologist who co-directs the UC Berkeley lab with Tjian, to scrutinize the phase separation literature. Later that year, in a December 2019 issue of *Genes and Development*, they published a scathing review of 33 studies that claimed to detect the process in cells. Tjian says he was "really disappointed by the quality of the papers." The evidence, he and his co-authors wrote, was "often phenomenological and inadequate to discriminate between phase separation and other possible mechanisms."

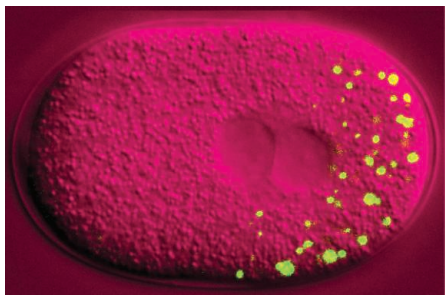
Too often, he and the other review authors asserted, researchers looking for phase separation rely on qualitative indicators—shape, for example—rather than quantitative data. Moreover, because many intracellular structures possibly formed by phase separation are so small, they are near what's known as the diffraction limit of traditional light microscopes. As a result, the structures may look like fuzzy orbs, but their real shape isn't discernible.

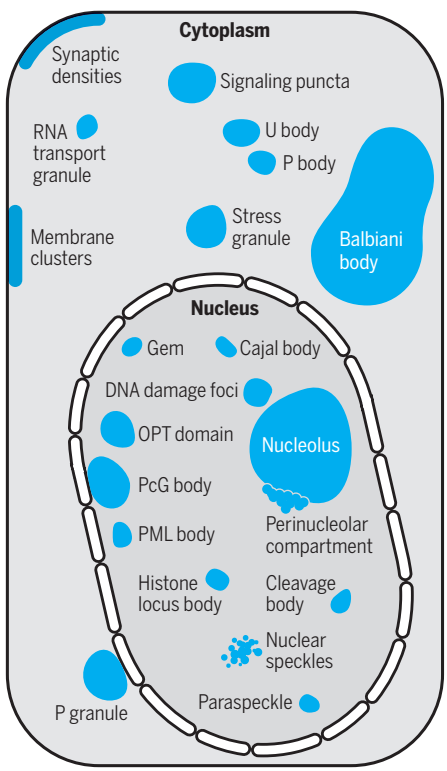
Tjian and colleagues also chastised researchers for often assuming the protein concentration in a cell is high enough to trigger phase separation, instead of actually measuring it. Overinterpretation "is rampant" in this type of research, Tjian says.

The scientists questioned the FRAP measurements that underpin many claims of phase separation. In the hands of different scientists, the group noted, FRAP recovery rates for the same molecule can range from less than 1 second to several minutes, indicating the technique is too variable to confirm phase separation. Darzacq adds that

## Dropping in

P granules (top, green), pockets of protein and RNA in early worm embryos that mark where sperm or egg cells will arise, have become the classic example of phase-separated regions in cytoplasm. But researchers propose that many other cellular features (bottom, blue), including some in the nucleus, form in the same way.






FRAP "only shows you have a liquid. You have liquid everywhere in the cell." Many of the congregations that researchers have identified with FRAP or other techniques are probably transient collections of molecules that only last a few seconds, Darzacq and Tjian say.

The review was "an invitation for all of us to proceed with a more careful and thoughtful in-depth analysis of cellular condensates," says molecular biophysicist Sua Myong of Johns Hopkins University. Although some scientists have been meticulous, "it has not been true of the field," Rosen adds.

Brangwynne says he, too, sees value in the critique. "I agree that we need quantitative approaches." For example, he concurs that researchers need to be more rigorous when interpreting imaging results so that "every diffraction-limited blob" isn't declared an example of phase separation.

Other recent papers have also raised doubts about cases of phase separation. In 2019 in *Non-Coding RNA*, Weber and a coauthor weighed the support for phase separation in the cell nucleus and concluded that solid data back its role in forming three structures, including the nucleolus, but not two other structures commonly attributed to the process.

And in April 2020 in *Molecular Cell*, biophysicist Fabian Erdel of the Center for Integrative Biology in Toulouse, France, and colleagues published a new investigation of heterochromatin—silenced regions of the genome in which DNA coils tightly with various proteins. Previous work suggested phase separation of the intracellular protein HP1 helped stretches of heterochromatin bunch up. But Erdel's team discovered that HP1 didn't form stable liquid droplets in mouse cells and that the size of the densely packed DNA regions didn't depend on the amount of the protein.

Brangwynne and other researchers argue that even if some individual findings cited by Tjian and colleagues remain in dispute, the field is making progress toward more solid results. To provide some of the rigor of test tube studies, he and his team have developed a technique for seeding cells with what they call corelets, combinations of molecular fragments that cluster when exposed to light. The corelets trigger droplet formation in cells, allowing the researchers to more precisely probe what protein concentrations are necessary for phase separation and which parts of the molecule are required for the behavior. Even Tjian and colleagues give the approach high marks.

Mir, who has been skeptical of much of the evidence for phase separation, agrees that the field seems to be moving away from the "everything is phase separation" stage to a more nuanced discussion of the formation and functions of condensates. "It's like any supertrendy thing in science. The noise subsides, and you are left with the truth."

To get to that truth, however, researchers "desperately need" new tools and a better understanding of the basic rules for how condensates form in cells, Gladfelter says. Scientists also need patience, she says, noting the field "tried to grow up and answer everything really fast." But she's confident researchers will eventually sort out the real importance of phase separation in cells. "Give us time. We'll get there." ■

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# Separation anxiety

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