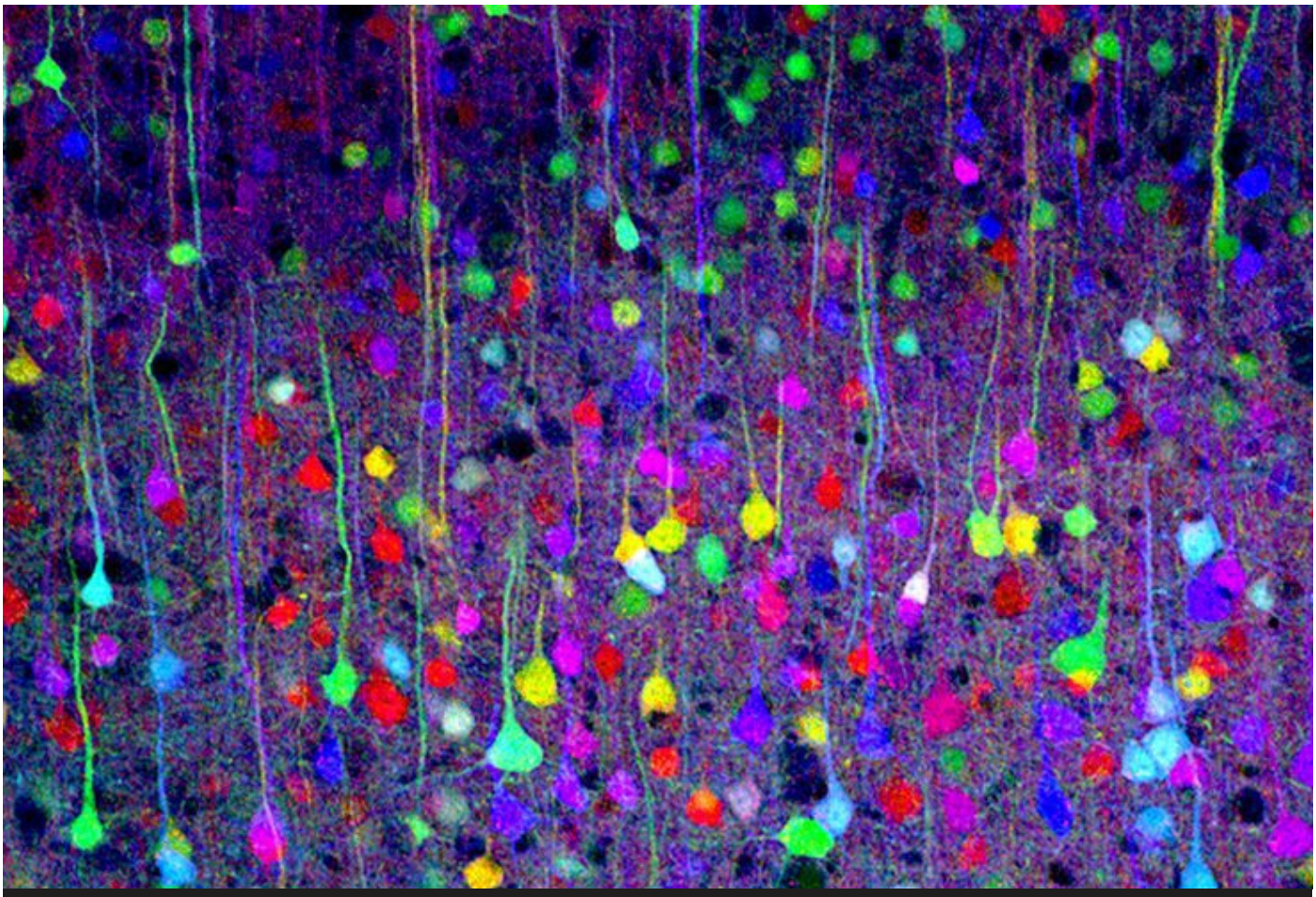
Nature, Meet Nurture

Single-cell analysis reveals dramatic landscape of genetic changes in the brain after visual stimulation

By KEVIN JIANG February 8, 2018



A "brainbow" of cerebral cortex neurons labeled with different colors. Image: Lichtman Lab/Harvard University

> *"Nature and nurture is a convenient jingle of words, for it separates under two distinct heads the innumerable elements of which personality is composed. Nature is all that a man brings with himself into the world; nurture is every influence from without that affects him after his birth." – Francis Galton, cousin of Charles Darwin, 1874.*

Is it nature or nurture that ultimately shapes a human? Are actions and behaviors a result of genes or environment?

Variations of these questions have been explored by countless philosophers and scientists across millennia.

Yet, as biologists continue to better understand the mechanisms that underlie brain function, it is increasingly apparent that this long-debated dichotomy may be no dichotomy at all.

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In a study published in ***Nature Neuroscience*** on Jan. 21, neuroscientists and systems biologists from Harvard Medical School reveal just how inexorably interwoven nature and nurture are.

Using novel technologies developed at HMS, the team looked at how a single sensory experience affects gene expression in the brain by analyzing more than 114,000 individual cells in the mouse visual cortex before and after exposure to light.

Their findings revealed a dramatic and diverse landscape of gene expression changes across all cell types, involving 611 different genes, many linked to neural connectivity and the brain's ability to rewire itself to learn and adapt.

The results offer insights into how bursts of neuronal activity that last only milliseconds trigger lasting changes in the brain, and open new fields of exploration for efforts to understand how the brain works.

"What we found is, in a sense, amazing. In response to visual stimulation, virtually every cell in the visual cortex is responding in a different way," said co-senior author **Michael Greenberg**, the Nathan Marsh Pusey Professor of Neurobiology and chair of the Department of Neurobiology at HMS.

"This in essence addresses the long-asked question about nature and nurture: Is it genes or environment? It's both, and this is how they come together," he said.

One out of many

Neuroscientists have known that stimuli—sensory experiences such as touch or sound, metabolic changes, injury and other environmental experiences—can trigger the activation of genetic programs within the brain.

Composed of a vast array of different cells, the brain depends on a complex orchestra of cellular functions to carry out its tasks. Scientists have long sought to understand how individual cells respond to various stimuli. However, due to technological limitations, previous genetic studies largely focused on mixed populations of cells, obscuring critical nuances in cellular behavior.

To build a more comprehensive picture, Greenberg teamed with co-corresponding author **Bernardo Sabatini**, the Alice and Rodman W. Moorhead III Professor of Neurobiology at HMS, and **Allon Klein**, assistant professor of systems biology at HMS.

Spearheaded by co-lead authors **Sinisa Hrvatin**, a postdoctoral fellow in the Greenberg lab, **Daniel Hochbaum**, a postdoctoral fellow in the Sabatini lab and **M. Aurel Nagy**, an MD-PhD student in the Greenberg lab, the researchers first housed mice in complete darkness to quiet the visual cortex, the area of the brain that controls vision.

They then exposed the mice to light and studied how it affected genes within the brain. Using technology developed by the Klein lab known as inDrops, they tracked which genes got turned on or off in tens of thousands of individual cells before and after light exposure.

The team found significant changes in gene expression after light exposure in all cell types in the visual cortex—both neurons and, unexpectedly, non-neuronal cells such as astrocytes, macrophages and muscle cells that line blood vessels in the brain.

Roughly 50 to 70 percent of excitatory neurons, for example, exhibited changes regardless of their location or function. Remarkably, the authors said, a large proportion of non-neuronal cells—almost half of all astrocytes, for example—also exhibited changes.

The team identified thousands of genes with altered expression patterns after light exposure, and 611 genes that had at least two-fold increases or decreases.

Many of these genes have been previously linked to structural remodeling in the brain, suggesting that virtually the

entire visual cortex, including the vasculature and muscle cell types, may undergo genetically controlled rewiring in response to a sensory experience.

There has been some controversy among neuroscientists over whether gene expression could functionally control plasticity or connectivity between neurons.

“I think our study strongly suggests that this is the case, and that each cell has a unique genetic program that’s tailored to the function of a given cell within a neural circuit,” Greenberg said.

Goldmine of questions

These findings open a wide range of avenues for further study, the authors said. For example, how genetic programs affect the function of specific cell types, how they vary early or later in life and how dysfunction in these programs might contribute to disease, all of which could help scientists learn more about the fundamental workings of the brain.

“Experience and environmental stimuli appear to almost constantly affect gene expression and function throughout the brain. This may help us to understand how processes such as learning and memory formation, which require long-term changes in the brain, arise from the short bursts of electrical activity through which neurons signal to each other,” Greenberg said.

One especially interesting area of inquiry, according to Greenberg, includes the regulatory elements that control the expression of genes in response to sensory experience. In a paper published earlier this year in ***Molecular Cell***, he and his team explored the activity of the FOS/JUN protein complex, which is expressed across many different cell types in the brain but appears to regulate unique programs in each different cell type.

Identifying the regulatory elements that control gene expression is critical because they may account for differences in brain function from one human to another, and may also underlie disorders such as autism, schizophrenia and bipolar disease, the researchers said.

“We’re sitting on a goldmine of questions that can help us better understand how the brain works,” Greenberg said. “And there is a whole field of exploration waiting to be tapped.”

Additional authors on the study include Marcelo Cicconet, Keiramarie Robertson, Lucas Cheadle, Rapolas Zilionis, Alex Ratner and Rebeca Borges-Monroy.

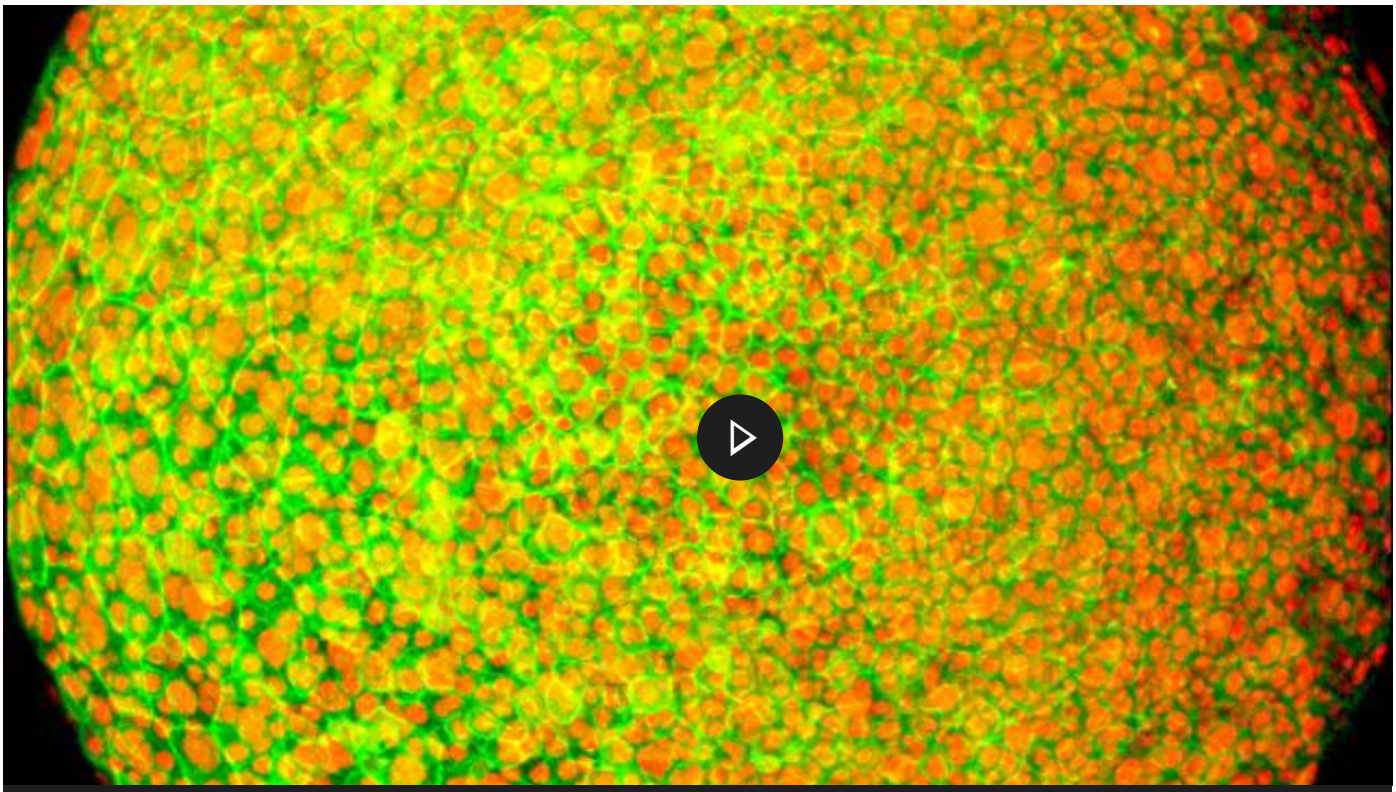
This work was supported by the National Institutes of Health (R01NS028829, R01NS046579, T32GM007753, R33CA212697, 5T32AG000222-23), F. Hoffmann-La Roche Ltd., the William F. Milton Fund, a Burroughs Wellcome Fund Career Award and an Edward J. Mallinckrodt Scholarship.

DOI:10.1038/s41593-017-0029-5

From One, Many

Harvard scientists reveal the genetic roadmap to building an entire organism from a single cell

By KEVIN JIANG April 26, 2018 | **Research**



A zebrafish egg cell forms a complex embryo in only a few hours. Video by Fengzhu Xiong and Sean Megason

Whether a worm, a human or a blue whale, all multicellular life begins as a single-celled egg.

From this solitary cell emerges the galaxy of others needed to build an organism, with each new cell developing in the right place at the right time to carry out a precise function in coordination with its neighbors.

This feat is one of the most remarkable in the natural world, and despite decades of study, a complete understanding of the process has eluded biologists.

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Now, in three landmark studies published online April 26 in *Science*, Harvard Medical School and Harvard University researchers report how they have systematically profiled every cell in developing zebrafish and frog embryos to establish a roadmap revealing how one cell builds an entire organism.

> "It is almost like going from seeing a few stars to seeing the entire universe" - Alexander Schier

Using single-cell sequencing technology, the research teams traced the fates of individual cells over the first 24 hours of the life of an embryo. Their analyses reveal the comprehensive landscape of which genes are switched on or off, and when, as embryonic cells transition into new cell states and types.

Together, the findings represent a catalog of genetic "recipes" for generating different cell types in two important model species and provide an unprecedented resource for the study of developmental biology and disease.

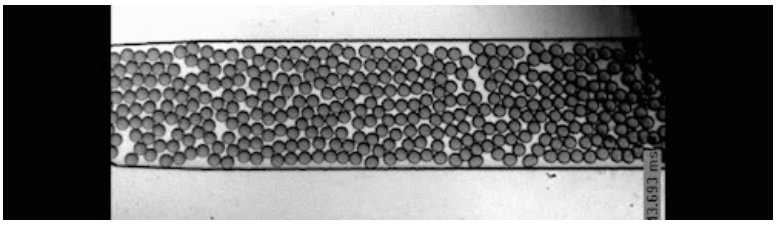
"With single-cell sequencing, we can, in a day's work, recapitulate decades of painstaking research on the decisions cells make at the earliest stages of life," said **Allon Klein**, HMS assistant professor of systems biology and co-corresponding author of two of the **three** ***Science*** **studies**.

Biomedically, these baseline resources for how organisms develop are as important as having baseline resources for their genomes, the researchers said.

"With the approaches that we've developed, we're charting what we think the future of developmental biology will be as it transforms into a quantitative, 'big-data'-driven science," Klein said.

In addition to shedding new light on the early stages of life, the work could open the door to a new understanding of a host of diseases, said **Alexander Schier**, the Leo Erikson Life Sciences Professor of Molecular and Cellular Biology at Harvard, and a corresponding author of the third study.

"We foresee that any complex biological process in which cells change gene expression over time can be reconstructed using this approach," Schier said. "Not just the development of embryos but also the development of cancer or brain degeneration."



Single-cell sequencing allows researchers to study gene expression in thousands of cells, one cell at a time. Video: Klein lab

One at a time

Every cell in a developing embryo carries within it a copy of the organism's complete genome. Like construction workers using only the relevant portion of a blueprint when laying a building's foundation, cells must express the necessary genes at the appropriate time for the embryo to develop correctly.

In their studies, Klein collaborated with co-authors **Marc Kirschner**, the HMS John Franklin Enders University Professor of Systems Biology, **Sean Megason**, HMS associate professor of systems biology and colleagues to analyze this process in zebrafish and western claw-toed frog (*Xenopus tropicalis*) embryos, two of the most well-studied model species in biology.

The researchers leveraged the power of **InDrops**, a single-cell sequencing technology developed at HMS by Klein, Kirschner and colleagues, to capture gene expression data from each cell of the embryo, one cell at a time. The teams collectively profiled over 200,000 cells at multiple time points across 24 hours for both species.

> "With these datasets, if someone wants to make a specific cell type, they now have the recipe" - Allon Klein

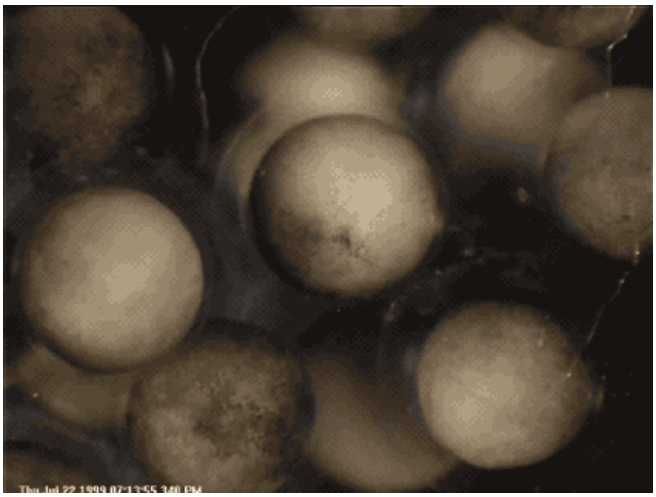
To map the lineage of essentially every cell as an embryo develops, along with the precise sequence of gene

expression events that mark new cell states and types, the teams developed new experimental and computational techniques, including the introduction of artificial DNA bar codes to track the lineage relationships between cells, called TracerSeq.

“Understanding how an organism is made requires knowing which genes are turned on or off as cells make fate decisions, not just the static sequence of a genome,” Megason said. “This is the first technological approach that has allowed us to systematically and quantitatively address this question.”

In the study co-led by Schier, the research team used **Drop-Seq**—a single-cell sequencing technology developed by researchers at HMS and the Broad Institute of MIT and Harvard—to study zebrafish embryos over 12 hours at high time resolution. Teaming with **Aviv Regev**, core member at the Broad, Schier and colleagues reconstructed cell trajectories through a computational method they named URD, after the Norse mythological figure who decides all fates.

Schier and colleagues profiled more than 38,000 cells, and developed a cellular “family tree” that revealed how gene expression in 25 cell types changed as they specialize. By combining that data with spatial inference, the team was also able to reconstruct the spatial origins of the various cells types in the early zebrafish embryo.


Developing Xenopus eggs. Courtesy Enrique Amaya

Recipe for success

In both species, the teams’ findings mirrored much of what was previously known about the progression of embryonic development, a result that underscored the power of the new approaches. But the analyses were unprecedented in revealing in comprehensive detail the cascades of events that take cells from early progenitor or “generalist” states to more specialized states with narrowly defined functions.

The teams identified otherwise difficult-to-detect details such as rare cell types and subtypes and linked new and highly specific gene expression patterns to different cell lineages. In several cases, they found cell types emerging far earlier than was previously thought.

> “The beauty of working on an organism is that this is it. Ten, 20 years from now, we can still be sure zebrafish and frogs are going to develop according to the same patterns" - Allon Klein

For scientists striving to answer questions about human disease, these data could be powerfully illuminating. In regenerative medicine, for example, researchers have for decades aimed to manipulate stem cells toward specific fates with the goal of replacing defective cells, tissues or organs with functional ones. Newly gleaned details about the sequence of gene expression changes that precipitate the emergence of specific cell types can propel these efforts further.

“With these datasets, if someone wants to make a specific cell type, they now have the recipe for the steps that those

cells took as they formed in the embryo," Klein said. "We've in some sense established a gold standard reference for how complex differentiation processes actually progress in embryos, and set an example for how to systematically reconstruct these types of processes."

When combined with one of the core concepts in biological inquiry—the idea of disrupting a system to study what happens—single-cell sequencing can yield insights difficult to attain before, Klein said.

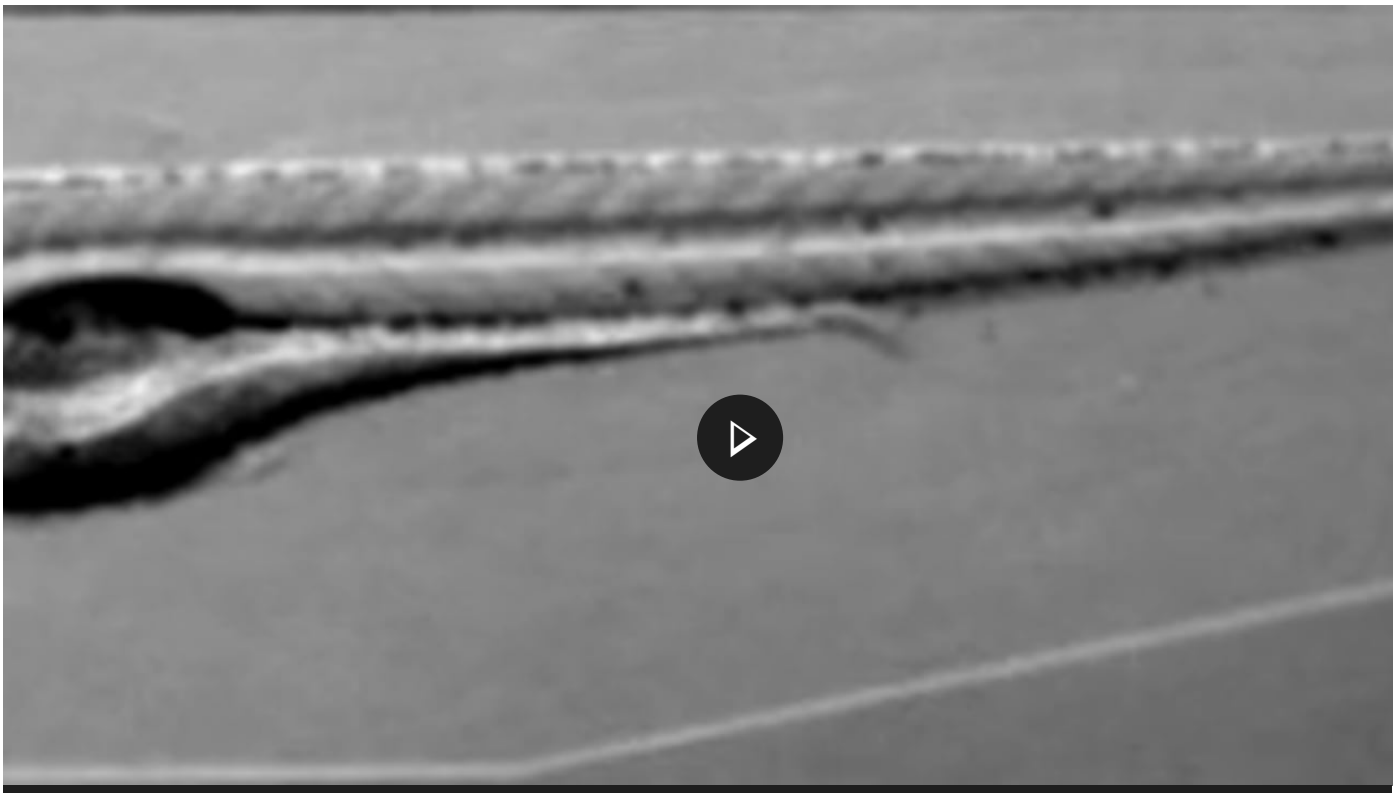
As a proof of principle, Klein, Megason and colleagues used the CRISPR/Cas9 gene editing system to create zebrafish with a mutant form of *chordin*, a gene involved in determining the back-to-front orientation of a developing embryo. Schier and colleagues took a similar approach by profiling zebrafish with a mutation in a different patterning gene known as *one-eyed pinhead*.

When analyzed with single-cell sequencing, the teams confirmed previously known descriptions of *chordin* and *one-eyed pinhead* mutants, and could describe in detail or even predict the effects of these mutations on developing cells and nascent tissues across the whole embryo.

Unexpectedly, the groups independently found that at the single-cell level, gene expression was the same in mutants and wildtype, despite the loss of an essential signaling pathway. The proportions of different cell types, however, changed.

"This work only became possible through recent technologies that let us analyze gene expression in thousands of individual cells," Schier said. "Now the scale is much larger, so that we can reconstruct the trajectory of almost all cells and all genes during embryogenesis. It is almost like going from seeing a few stars to seeing the entire universe."

Rethinking definitions



A zebrafish embryo develops. Video by Ian Swinburne and Sean Megason

The research teams also demonstrated how these data can be mined to answer long-standing fundamental questions in biology.

When Klein, Kirschner, Megason and colleagues compared cell-state landscapes between zebrafish and frog embryos, they observed mostly similarities. But their analyses revealed numerous surprises as well. One such observation was

that genes marking cell states in one species were often poor gene markers for the same cell state in the other species.

In several instances, they found that the DNA sequence of a gene—and the structure of the protein it encodes—could be nearly identical between species but have very different expression patterns.

"This really shocked us, because it goes against all the intuition we had about development and biology," Klein said. "It was a really uncomfortable observation. It directly challenges our idea of what it means to be a certain 'cell type.'"

The reason that these differences were not spotted before, the researchers hypothesize, is that computational analyses "pay attention" to data in a way fundamentally different from how humans do.

"I think this reflects some level of confirmation bias. When scientists find something conserved between species, they celebrate it as a marker," Megason said. "But often, all the other nonconserved features are ignored. Quantitative data helps us move past some of these biases."

In another striking finding, the teams observed that the process of cell differentiation into distinct cell types—which is commonly thought to occur in a tree-like structure where different cell types branch off from a common ancestor cell—can form "loops" as well as branches.

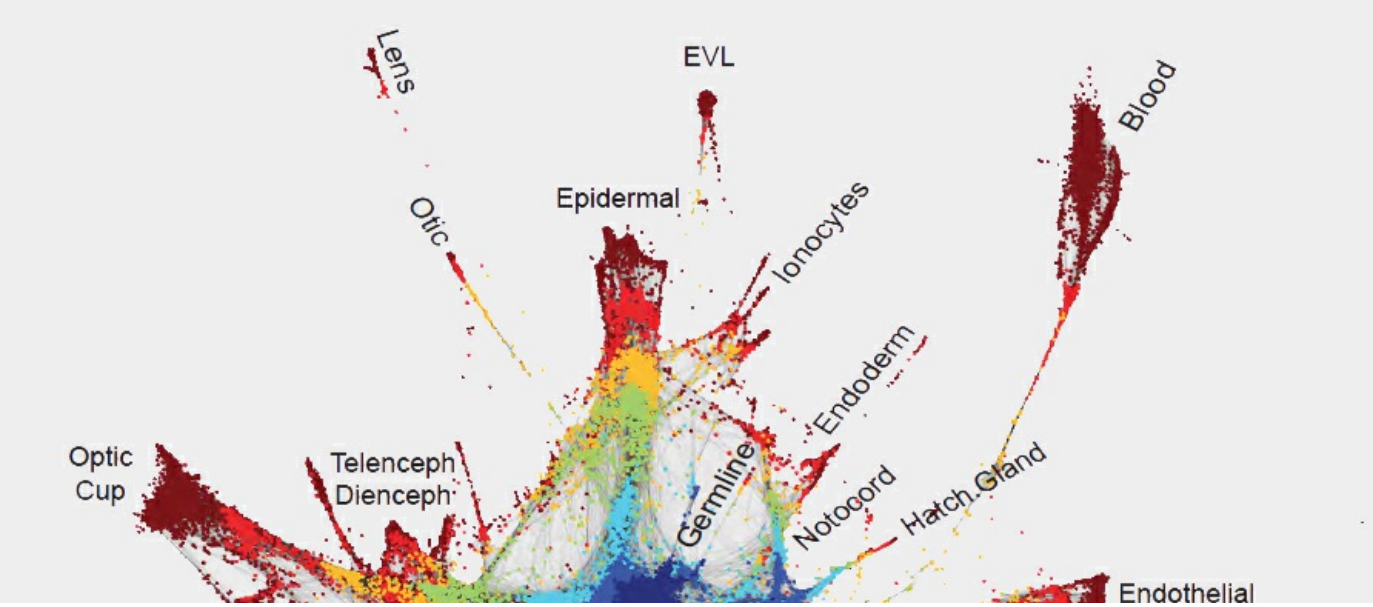
For example, the neural crest—a group of cells that give rise to diverse tissue types including smooth muscle, certain neurons and craniofacial bone—initially emerges from neural and skin precursors, but is well-known to generate cells that appear almost identical to bone and cartilage precursors.

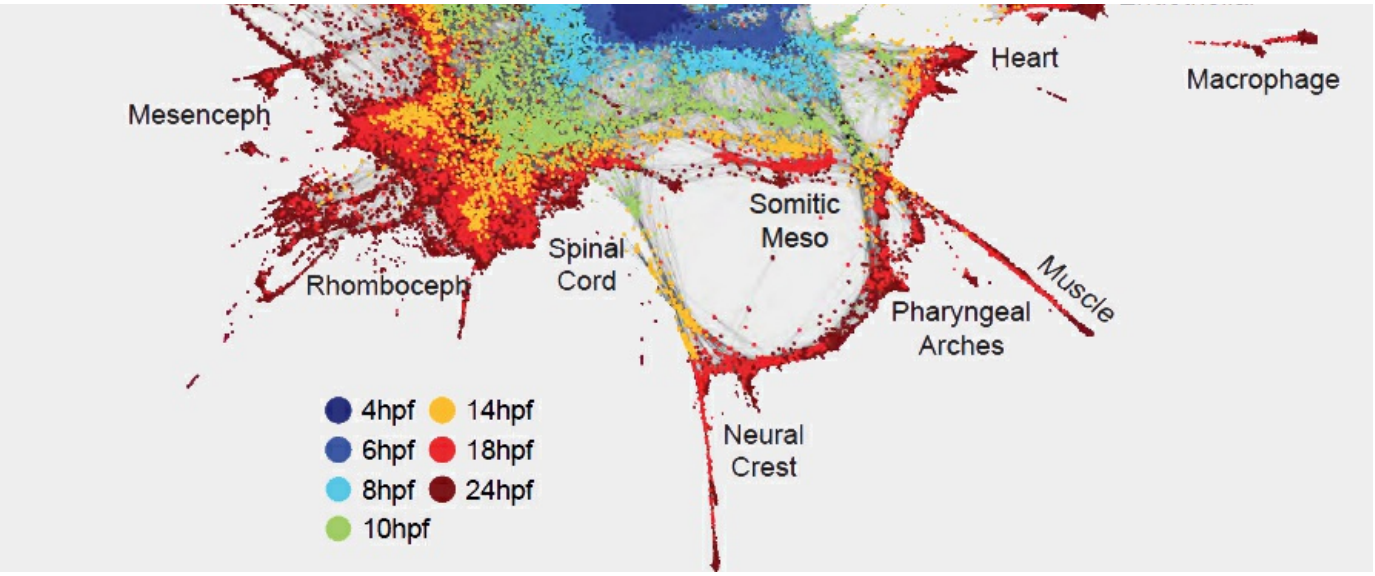
The new results suggest that similar loops might occur in other situations. That cells in the same state can have very different developmental histories suggests that our hierarchical view of development as a "tree" is far too simplified, Klein said.

All three teams also identified certain cell populations that existed in a kind of intermediate "decision making" state. Schier and colleagues found that, at certain key developmental branch points, cells appeared to go down one developmental trajectory but then changed their fate to another trajectory.

Klein, Megason, Kirschner and colleagues made a related observation that, early in development, some cells activated two distinct developmental programs. Though those intermediate cells would eventually adopt a single identity, these discoveries add to the picture of how cells develop their eventual fate and hint that there may be factors beyond genes involved in directing cell fate.

"With multilineage cells, we have to start wondering if their final fate is being determined by some selective force or interaction with the environment, rather than just genetic programs," Kirschner said.





A new view of embryonic development, showing 50,000+ cells organized by relatedness over time. The blue center is the beginning of life. Radiating outwards, specialized cell types emerge. Image: Wagner et al.

Future foundation

The newly generated data sets and the new tools and technologies developed as part of these studies lay the foundation for a wide spectrum of future exploration, according to the authors.

Developmental biologists can gather more and higher quality data on many species, follow embryos further in time and perform any number of perturbation experiments, all of which can help improve our understanding of the fundamental rules of biology and disease.

These resources can also serve as a focal point for collaboration and interaction since most labs do not have the depth of expertise needed to exploit all the data and information generated, the authors noted.

"I think these studies are creating a real sense of community, with researchers raising questions and interacting with each other in a way that harkens back to earlier times in the study of embryology," Kirschner said.

The three studies, Schier said, are an example of how the scientific community can work on complementary questions to answer important questions in biology.

"Instead of competing, our groups were in regular contact over the past two years and coordinated the publication of our studies," he said. "And it is great how complementary the three papers are—each highlights different ways such complex data sets can be generated, analyzed and interpreted."

The next conceptual leap, the teams suggest, will be to better understand how cell-fate decisions are made.

"Right now, we have a roadmap, but it doesn't tell us what the signs are," Megason said. "What we need to do is figure out the signals that direct cells down certain roads, and what the internal mechanisms are that allow cells to make those decisions."

Whatever the future holds, these data sets will leave their mark.

"The beauty of working on an organism is that this is it," Klein said. "Ten, 20 years from now, we can still be sure zebrafish and frogs are going to develop according to the same patterns."

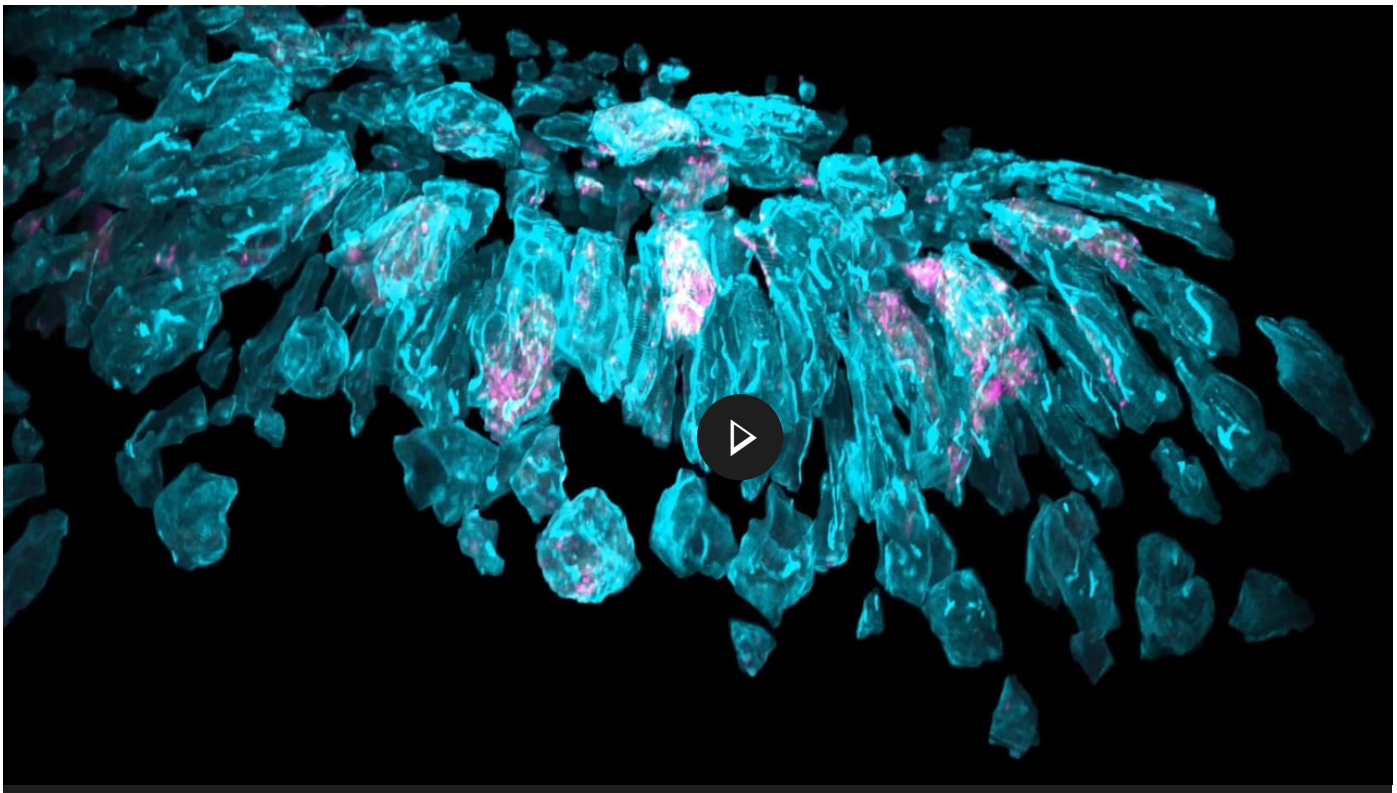
All three research teams have made their data sets and tools available as interactive, browsable online resources. For Klein and colleagues: **zebrafish** and ***Xenopus***. For Schier and colleagues: **zebrafish** and **URD**.

News & Research

To Boldly Go

New microscope captures 3-D movies of cells inside living organisms in unprecedented detail

By KEVIN JIANG | April 19, 2018 **Research**



New microscope technology allows us to see cells like we've never seen them before. Video: Rick Groleau and Kevin Jiang

More than 350 years ago, the English natural philosopher Robert Hooke peered through a microscope at a thin slice of cork and discovered that it was made of small box-like compartments, which he named "cells."

From that moment on, Hooke and countless inquisitive minds after him strived to gain a better view of these fundamental building blocks of life.

Now, our window into the cellular world has become a lot clearer.

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In a new study in the April 20 issue of ***Science***, researchers from Howard Hughes Medical Institute's (HHMI) Janelia Research Campus, Harvard Medical School and collaborating institutions report the development of a microscope capable of capturing 3-D images and videos of cells inside living organisms in unprecedented detail.

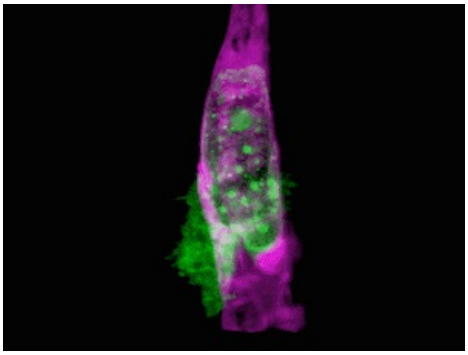
> "It's like 'Star Trek.' It's the age of exploration again." - Gokul Upadhyayula, HMS instructor of pediatrics

Adapting a technique used by astronomers to study distant stars, the research team, led by Nobel laureate and Janelia group leader **Eric Betzig**, showcased the new technology by generating a series of stunning movies: cancer cells crawling through blood vessels, spinal nerve cells wiring up into circuits, immune cells cruising through a zebrafish's inner ear and much more.

The resolution of the microscope is powerful enough to even capture subcellular details such as the dynamics of miniscule bubbles known as vesicles, which transport molecular cargo through to the cell.

"This is the miracle of being able to see what we have never been able to see before. It's simply incredible," said study co-author **Tomas Kirchhausen**, HMS professor of cell biology, the Springer Family Chair of pediatrics and a senior investigator at Boston Children's Hospital.

"Every time we've done an experiment with this microscope, we've observed something novel—and generated new ideas and hypotheses to test," Kirchhausen said. "It can be used to study almost any problem in a biological system or organism I can think of."



A cancer cell (green) forces its way through the wall of a blood vessel (purple)

While scientists have used microscopes to look at cells for centuries, the clearest views thus far have come from cells isolated on glass slides. Visualizing living cells in real time inside live organisms has remained far more challenging.

Cells of interest are surrounded by tissues and other biological structures that scramble light coming from and returning to a microscope objective, which blurs and obscures important details. Light powerful enough to penetrate biological structures and yield a crisper view of cells, on the other hand, can damage tissues.

"This raises the nagging doubt that we are not seeing cells in their native state, happily ensconced in the organism in which they evolved," said Betzig, who is corresponding author on the study. "It's often said that seeing is believing, but when it comes to cell biology, I think the more appropriate question is, 'When can we believe what we see?'"

Guide star

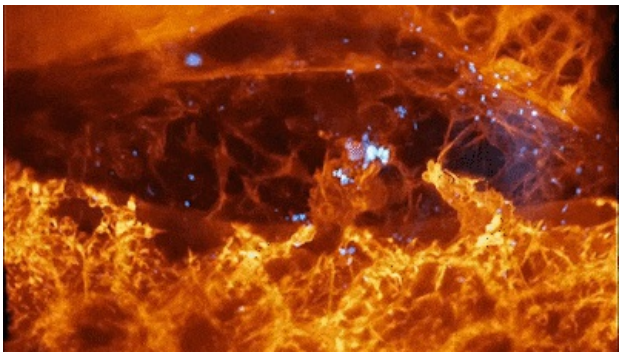
To address these challenges, Betzig and colleagues combined two technologies: lattice light sheet microscopy, which Betzig developed in the early 2010s, and adaptive optics, a technique borrowed from astronomy.

Lattice light sheet microscopy uses rapid and repeated sweeps of an ultrathin sheet of light, which avoids the bleaching or damage associated with traditional focused beams of light. It can be used to generate high-resolution 2-D images of living cells as they carry out their functions, and by combining series of these images over time, scientists can create 3-D movies.

To unscramble the light sheet as it passes through tissues and other structures, the research team turned to the stars. To see distant objects through the Earth's atmosphere, astronomers rely on adaptive optics—deformable mirrors and light modulators.

The process uses a powerful laser, aimed at the small region of the sky they want to image, which serves as a "guide star." As the laser passes through the atmosphere, optical aberrations that distort its path are revealed and corrected by the adaptive optics.

Betzig and colleagues applied this principle to the microscopic world, using a two-photon laser to create an adaptive optics system that maintains the thin illumination of a lattice light sheet as it penetrates an organism to generate distortion-free images of their target of interest.



Neutrophils (center) gobble up color-labeled sugars (blue) in a developing zebrafish eye. Blood cells (top) zoom through a capillary.

The team validated the new adaptive optics-lattice light sheet microscope on a variety of biological samples, much of it carried out through the laboratories of Kirchhausen and **Sean Megason,** HMS associate professor of systems biology.

To make sense of the data they generated, the team created bespoke software and computational and visualization workflows, spearheaded by study co-lead authors **Gokul Upadhyayula,** HMS instructor in pediatrics at Boston Children's and research associate in the Kirchhausen lab, and Tsung-Li Liu, formerly a research scientist in the Betzig lab at HHMI.

"For the types of data we generated, there's no one commercial software that we can use to create interpretable movies and extract biologically meaningful information, so we built the necessary tools," Upadhyayula said. "This allowed us to understand what we acquired and visualize the data in meaningful ways, including, in the near future, fully interactive 3-D movies."

Into focus

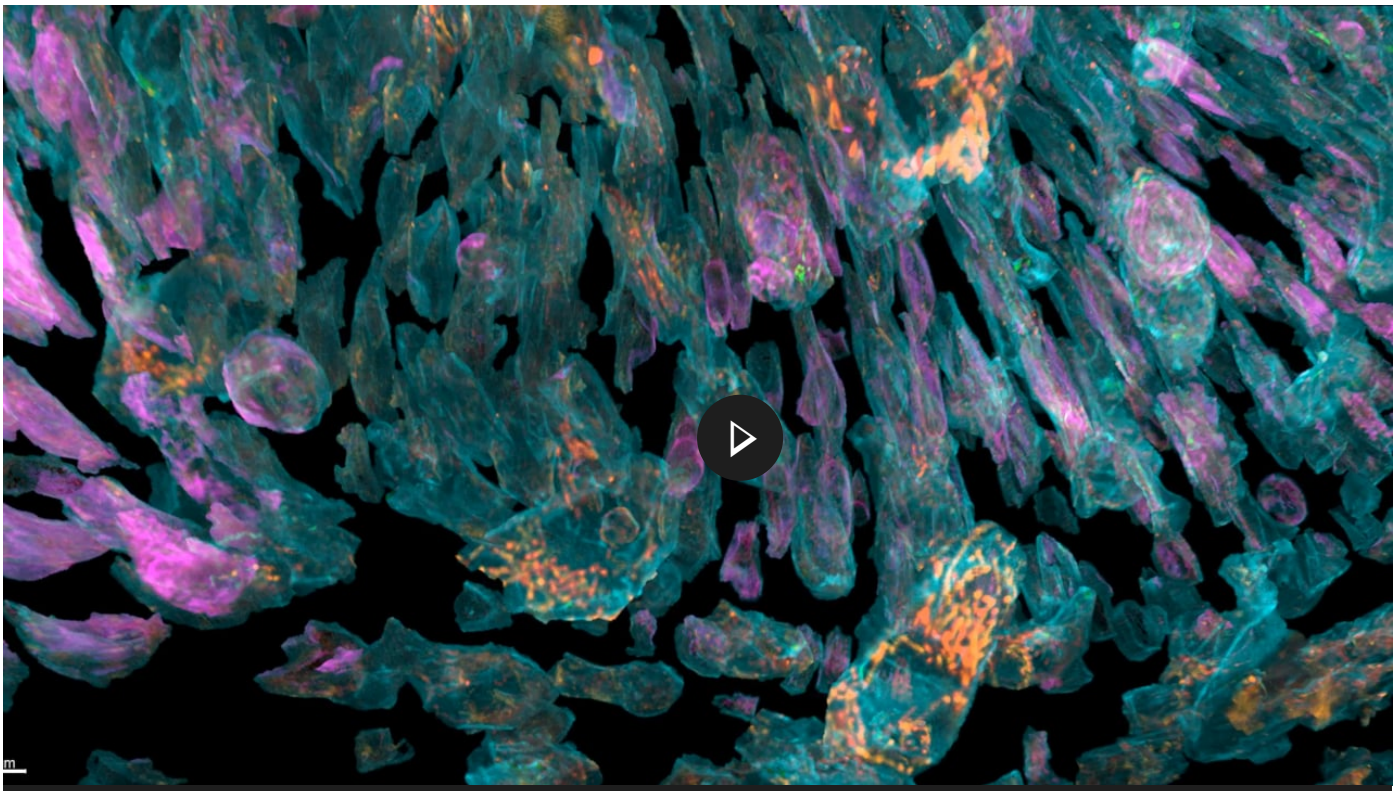
The results were remarkable. In one movie, a fiery orange immune cell wriggles madly through a zebrafish's ear while scooping up blue sugar particles along the way. In another, a migrating cancer cell trails sticky appendages as it rolls through a blood vessel and attempts to squeeze through the vessel wall.

The team captured movies of the behavior of organelles as they remodel themselves inside cells during cell division and could even visualize in real-time and at near-molecular detail the process of **clathrin-mediated endocytosis,** which cells use to capture materials from their exterior environment.

"I work on understanding how cells 'eat' using machinery based on vesicular carriers, and all my life I've dreamed of seeing this in a live organism," Kirchhausen said. "We have finally achieved this."

The complexity of the 3-D multicellular environment can be overwhelming, Betzig said, but the clarity of the imaging allows them to computationally "explode" apart the individual cells in tissue to focus on the dynamics within any particular one.

"It's like 'Star Trek.' It's the age of exploration again," Upadhyayula said. "We don't even know what questions to ask yet because we've never even seen some of these biologies at this level of detail."



Cells of a zebrafish eye, computationally "exploded"

All this detail is hard to see without adaptive optics, Betzig said. “It’s just too damn fuzzy.” In his view, adaptive optics is one of the most important areas in microscopy research today, and the lattice light sheet microscope, which excels at 3-D live imaging, is the perfect platform to showcase its power.

The next step is making the technology affordable and user friendly. The current microscope takes up a 10-foot-long table. In collaboration with Kirchhausen and Upadhyayula, Betzig’s team is working on a next-generation version that should fit on a small desk at a cost within the reach of individual labs.

The first such instrument will go to Janelia’s Advanced Imaging Center, where scientists from around the world can apply to use it. A second instrument built at the same time will be located in the Kirchhausen laboratory at the HMS Quad in Boston. Plans to build the instrument will also be made available.

Contributing authors include researchers from Stony Brook University, University of California, Berkeley, California Institute of Technology and the University of Exeter.

The study was supported by the Howard Hughes Medical Institute, National Institutes of Health (R01GM075252, R01DC015478, 5R00CA154870-05, 1R01GM121597-01, R01CA196884 and R35GM118149), National Science Foundation (IOS1452928), Carol M. Baldwin Breast Cancer Research Fund, Damon Runyon Cancer Research Foundation, Pew Charitable Trusts, Biogen, Ionis Pharmaceuticals and a Human Frontier Science Program fellowship.