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Webinar
Innovations in Light Sheet Microscopy
Strategies and New Applications

Speakers

Thai Truong, Ph.D.
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Los Angeles, CA

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Wednesday, October 29, 2014
12 noon Eastern, 9 a.m. Pacific
4 p.m. UK (GMT), 5 p.m. Central Europe (CET)

Over the past 10 years, light sheet microscopy (or selective plane illumination microscopy, SPIM) has transformed the microscopy field, offering a faster, less phototoxic technique than conventional methods that can create true 3-D images. Ideal for observing living organisms and the cellular dynamics of biological systems, this method uses a unique illumination approach to achieve high penetration depths, fast imaging speeds, and subcellular-level resolution. Because a specimen is illuminated with a sheet of light rather than a focused laser beam, only regions directly exposed to light will fluoresce—creating minimal photo-induced tissue damage.

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Rewriting the Genome: Even DNA Needs an Editor

Until relatively recently, the power of molecular biology was at once vast and limited. Researchers who wanted to knock out specific genes to see what they did mostly had to restrict such studies to mice, and specific strains at that. Now a new class of genome- and epigenome-editing tools is reshaping the landscape. From Arabidopsis to humans to zebrafish, researchers are finding that, generally speaking, when it comes to the genome, if they can dream it, they can build it. By Jeffrey M. Perkel

To get a sense of the transformative power of genome-editing tools, consider plants. Unlike mice, there is no such thing as a plant embryonic stem (ES) cell. Even if there were, homologous recombination rates in plant cells are too low to yield reliable genetic modification without a little help. Thus, genetic tinkering in these organisms is typically accomplished using either random mutagenesis or genetic crosses, or via Agrobacterium infection or a specialized “gene gun.”

Such techniques have been used successfully, of course—most crop plants these days are genetically modified. But those efforts rely on random events; plant researchers had no way to make targeted genomic modifications, explains Dan Voytas, professor of genetics and director of the Center for Genome Engineering at the University of Minnesota, Minneapolis.

Now, thanks to genome engineering technology, they do. “You can say, okay, I know what that gene is, I know the sequence variation I need to introduce into my crop plants, and you can just go ahead and introduce it directly without engaging in an extensive breeding program,” Voytas says.

On the research front, such tools are nothing short of transformative. Voytas uses them to rewrite the genome in plants such as tobacco and tomato, and they hold similar promise for other model organisms. In the clinic, genome editing can be coupled with human induced pluripotent stem cell technology to, for instance, create genetically repaired patient-specific transplants.

Questions of targeting specificity remain, but Phillip Sharp, institute professor at the Koch Institute for Integrative Cancer Research at the Massachusetts Institute of Technology (MIT), calls these technologies a “game-changer” on par with RNA interference. “RNAi was a major transition in how we do cell biology; this will be something comparable. And it’s going to greatly accelerate the speed in which we can probe problems from cancer to other normal development using our biological systems. It’s really a very important advance.”

Nuclease-based strategies

Genome-engineering approaches generally use a site-specific endonuclease to introduce a double-stranded DNA break at a specified point in the genome, like a custom restriction enzyme. As the cell repairs the break, it can either inadvertently disrupt the gene by adding or removing a few bases (a process called nonhomologous end-joining, or NHEJ) or use an exogenous piece of donor DNA to rewrite the damaged sequence to researchers’ specifications via homologous recombination.

One of the earliest nuclease technologies involved enzymes called meganucleases, but these were less than user-friendly, explains Philippe Duchateau, chief scientific officer at Cellectis, a French genome-engineering firm that still uses the technology. “They are very difficult to engineer ... [requiring] a long and costly process.”

Zinc finger transcription factors have proven easier to manipulate. Researchers have long known that these proteins bind to DNA in a modular fashion, with each “finger” recognizing three to four specific nucleotides. In 2003, two teams independently demonstrated that they could string together custom arrays of zinc fingers to target novel DNA sequences and couple the array to the FokI nuclease to induce specific DNA changes at those sites. One group knocked out the yellow gene in fruit flies; the other repaired a GFP mutation in human cells. Two years later, researchers at Sangamo BioSciences used these so-called zinc finger nucleases (ZFNs) to repair a mutation in the IL2R-gamma gene in human cells, demonstrating the clinical potential of the technology.

Yet ZFNs never really took off in academia, in part because the technology was largely inaccessible—Sangamo owned the intellectual property, though they licensed it to Sigma Aldrich in 2007 for research purposes—and partly because good ZFNs are hard to build. In theory, explains Philip Gregory, senior vice president and chief scientific officer at Sangamo BioSciences, a library of 64 zinc fingers (one for each codon) should be sufficient to target any sequence. But in practice, a given finger’s binding properties differ from
array to array. “Context matters,” he says.

In 2009, researchers deciphered a new class of transcription factors called TALEs. Like ZFNs, TALEs are modular structures, but each TALE module specifies a single nucleotide in the recognition sequence, and does so more or less independently of its neighbors. By fusing custom TALEs to the FokI nuclease (a so-called TALE nuclease, or TALEN), researchers could target pretty much any sequence they desired—though as TALENs are substantially larger than ZFNs and highly repetitive, the cloning process itself isn’t trivial (TALEN research tools are available through Addgene and Life Technologies, part of Thermo Fisher Scientific.)

TALENs made genome-editing technology accessible in a way it hadn’t been before. But in 2012 the editing landscape tilted on its axis again when Jennifer Doudna, a Howard Hughes Medical Institute (HHMI) investigator and professor of biochemistry and molecular biology at the University of California, Berkeley, and Emmanuelle Charpentier, then at Umeå University, worked out the mechanics of an RNA-guided bacterial immune system complex called clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9.

With reagents available through Addgene, Horizon Discovery, Life Technologies, New England Biolabs, and Sigma Aldrich, the two-component CRISPR-Cas9 is by far the simplest genome-editing system to date. It takes at least a week to make a TALEN, and sometimes months for a ZFN. But all CRISPR-Cas9 requires is a 20-nucleotide “single-guide” RNA specifying the desired target and Cas9 nuclease to cut it. That makes it easy for neophytes to implement the technology, and to explore different targeting sites. And, because its sequence targeting is encoded in RNA rather than protein, Cas9 can hit multiple sites at once, a process known as multiplexing.

Voytas likens genome-editing approaches to the evolution of DNA sequencing. “ZFNs are sort of [like] Maxam–Gilbert—you can get it to work and you can get the information, but you struggled to do so. TALENs are [like] Sanger sequencing. And next-generation sequencing is [akin to] CRISPR-Cas, it just happens so fast and easily,” he says.

Indeed, William Skarnes, a senior group leader at the Wellcome Trust Sanger Institute in Cambridge, United Kingdom, has spent more than a decade knocking out genes in mice the old-fashioned way. Between creating the targeting vectors, inserting them into ES cells, testing for gene modification, inserting the cells into blastocysts, and testing and crossing founders, each line could take from six to 12 months to create, he says. Now, that library is looking dated. “With CRISPR technology it’s just a simple injection of some very simple reagents directly in a one-cell embryo to create the same mutations. It’s remarkable—I mean, it’s something that we dreamt about at the very beginning of ES cell technologies.”

Targeting specificity

That’s not to say CRISPR-Cas9 is without flaws. At least in its initial incarnation, the system is less discriminating than users would like, and single-guide RNAs have been shown to induce off-target effects in the human genome. Naturally, researchers have been developing strategies to counteract that problem.

Some, like Harvard University geneticist George Church, have shown that knocking out one of the two DNA cutting sites in Cas9 to create a so-called nickase, and pairing those molecules using two closely spaced guide RNAs, dramatically improves specificity. (That strategy is being commercialized by Sigma Aldrich, according to principal R&D scientist Greg Davis, who notes the approach works with guides as far as 150 bases apart. “This is a key distinguishing point,” he says, as it provides considerable targeting flexibility.) J. Keith Joung, associate chief of pathology for research at Massachusetts General Hospital, and David Liu at Harvard University independently described another approach, fusing a catalytically inactive Cas9 to FokI. Because FokI is an obligate dimer, that construction, like paired nickases, requires two binding events and thus yields greater precision, albeit with less targeting flexibility.

Guide RNA length can also influence specificity. Some have shown that extending the guide RNA by two nucleotides reduces off-target effects, while in Joung’s hands truncating the guide RNA reduces off-target mutation rates by up to 5,000 fold.

What is lacking, says Joung, is a side-by-side comparison to see which strategy truly performs best, not to mention a method—other than whole-genome sequencing—to identify modified sites across the genome. Still, Church says the technology—with an error rate of about one in 100 billion base pairs—probably is already sufficiently specific for research applications, where simple expedients such as comparing the effects of multiple guide RNAs can likely overcome possible off-target effects. Rather, it is in clinical applications where specificity really counts, he says, and the only way to determine if that’s an issue is to put the technology into animals, and ultimately humans, and see what happens.

“In the end it’s not going to be determined by sequencing off-targets,” he says. “It’s going to be determined by whether animals get cancer or not.”

Clinical applications

In early 2014, Sangamo published the first human clinical trial to use genome-editing technology. The company’s researchers harvested CD4+ T cells from HIV+ patients, infected them with a virus expressing a ZFN to knock out the HIV co-receptor CCR5 via NHEJ, and returned them to patients. “In every case, on infusion of the CD4 T cells, we see a marked..."
increased in CD4 T cells in these patients,” says Gregori. Furthermore, those cells exhibited a “selective survival advantage” relative to unmodified cells, he says.

Sangamo is also pursuing homologous recombination-based therapeutics, says Gregori. For instance, its researchers recently published data demonstrating the ability to repair the IL2R-gamma gene in hematopoietic stem cells from patients with X-linked severe combined immunodeficiency—a cell population that theoretically could be used to reseed a patient’s bone marrow.

TALENs and CRISPRs also are heading to the clinic, with Collectics pursuing the former and Editas Medicine and CRISPR Therapeutics the latter.

In June, Daniel Anderson and Tyler Jacks at MIT demonstrated the potential clinical power of CRISPR-Cas9-based editing by delivering a vector co-expressing Cas9 and a guide RNA, plus a second DNA containing a repair template, into the tail veins of a mouse model of hereditary tyrosinemia type I, which is caused by a single point mutation in the fumarylacetoacetate hydrolase gene. Injection corrected the mutation in 1 out of every 250 liver cells, rescuing the diseased phenotype within a month. “I thought that was just amazing,” Doudna says.

Epigenome editing

Still, for all its potential as a nuclease, Cas9 can do so much more. The protein is just a “molecular machine that recognizes DNA in a targeted way,” Doudna says. As a result, it, and TALEs and zinc fingers for that matter, can serve as platforms for more extensive genomic tinkering—“epigenome engineering,” as it were.

Researchers have coupled catalytically inactive Cas9 and TALE domains to transcription activators and repressors, DNA methyltransferases, and histone modifying enzymes, thereby directing those activities to specific sites in the genome. They have coupled TALENs and Cas9 to fluorescent proteins to investigate chromosomal architecture. Church’s team has even identified Cas9 orthologs with different sequence requirements, which could be used, among other things, to target distinct activities to different genomic loci simultaneously.

Such epigenomic designs have potential clinical value, says Feng Zhang, core member of the Broad Institute of MIT and Harvard and a co-founder of Editas. “From a therapeutic perspective you may want to turn a gene on that needs to be on but for some epigenetic reason is turned off,” he says. For example, there are inherited diseases in which the maternal (expressed) copy of a gene is mutated while the paternal copy is wild type but silent. “One way to treat it might be to turn that gene on by using a Cas9 or TALEN activator,” Zhang says.

But such strategies present their own complications, especially regarding off-target effects. In one recent study, a team led by Sharp and Zhang used chromatin immunoprecipitation and next generation DNA sequencing (ChIP-Seq) to determine where in the genome a catalytically inactive but RNA-guided Cas9 protein binds. The team identified between 2,000 and 20,000 binding sites per guide RNA tested. But when they used a catalytically active Cas9, only one of 295 potential off-target sites the team tested was actually modified, suggesting that sequence binding and cutting occur in two distinct steps.

“This is a non-trivial nuance,” explains Stephen Ekker, professor of biochemistry and molecular biology at the Mayo Clinic Cancer Center in Rochester, Minnesota. “There are many reviews that argue that what happens is that the guide RNA finds double-stranded DNA in an extended complex, like a PCR primer does, and then Cas9 comes in and cuts it. That’s not how it works.” Sharp’s data instead suggest a genome-scanning mechanism in which binding and cutting are distinct events—an observation that implies epigenetic control using dead Cas9 fusions could have unintended consequences, as the protein may bind sites other than its intended targets. “Basically, any approach that really depends on having a single specific binding event may be compromised,” he says.

Still, there’s no denying the power of CRISPR-Cas9, and other editing tools. Ekker, who teaches a course on genome engineering, says molecular biology could be on the cusp of a technology boom akin to that ushered in by the invention of the transistor. Only time will tell what novel applications arise, he says. But this at least is clear: Researchers need no longer think of the genome as something with which they “tinker;” they can, well, engineer it.

Jeffrey M. Perkel is a freelance science writer based in Pocatello, Idaho. DOI: 10.1126/science.opms.p1400089
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