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BIOLOGICAL INSIGHTS INTO SINGLE-MOLECULE IMAGING

Interview with Professor Eric Betzig

Dr. Eric Betzig *is a Professor of Cell and Developmental Biology, Eugene D. Commins Presidential Chair in Experimental Physics, and a Howard Hughes Medical Institute Investigator at the University of California, Berkeley. He is also a Senior Fellow at the Janelia Research Campus. In 2014, Professor Betzig was awarded the Nobel Prize in Chemistry for his development of super-resolution fluorescence microscopy. Professor Betzig's research centers on advancing imaging tools for biological discovery in dynamic living systems. In this interview, we discuss the foundations of fluorescence microscopy, challenges associated with live-cell imaging, and the project of mapping nanoscale synaptic proteins across the entire Drosophila brain.*

BSJ: You have a very diverse background, ranging from physics to hydraulics engineering to optical microscopy. Can you tell us about how you came to working on developing optical imaging tools for biological research?

EB: By accident, I guess. I went to Caltech as an undergrad in the late seventies. I originally wanted to be an astronaut or an astrophysicist, but I realized that was really hard and probably beyond what I could do. I started working in a lab and I liked doing experimental work, so I switched gears toward applied physics and engineering. Back then, the only two graduate schools that had applied physics programs were Stanford and Cornell. I had had enough of California at that point, so I moved to Cornell. The department was really small; there were only about 12 professors, and two were young associate professors who had just gotten tenure. One was an electron microscopist and the other was a Raman spectroscopist. Together, they had come up with this crazy idea to use an electron beam to drill a hole into a silicon film and shine light through it, making a "nano-flashlight" that could be driven around on a sample. That was called near-field microscopy. What these two professors were doing sounded kind of nutty and fun, so I got involved in microscopy that way. I did near-field microscopy at Cornell for six years and then got a job at Bell Labs, where I did the same thing in my own lab for another six years.

BSJ: Why is diffraction-unlimited microscopy useful for studying biological systems?

EB: We've learned a lot about biology without going beyond the diffraction limit, but the main problem with the diffraction limit is that at the fundamental level, cells are made of molecules. The resolution for a normal optical microscope is 100 times too coarse to see what's going on at the molecular level. We'd like to understand how one inanimate molecule interacts with other inanimate molecules to somehow make a cell, which can move, excrete, reproduce, and is deterministic. You want a microscope that can get down to the molecular resolution if you want to understand how the cell works.

BSJ: What does it mean to image "fixed" cells? How do fixation procedures introduce artifacts in imaging?

EB: Fixation is a sort of secret home brew that's been developed over many generations. There are all sorts of recipes, but typically chemicals like formaldehyde and glutaraldehyde are used to cross-link proteins together. At that point, you have a static cell. But with the way the proteins are cross-linked, cellular structures can be distorted. This distortion isn't so bad if you're looking at tissues at low resolution. But with super-resolution microscopes, you realize that cells look like roadkill after they've been fixed. The real fun in biology is looking at living, moving things.

BSJ: Super-resolution microscopy methods center on detecting fluorescence signals from chemical compounds called fluorophores. Can you explain what fluorescence is and how it is useful for single-molecule studies?

Image: Professor Eric Betzig.[1] *Giving a talk at a conference at École Polytechnique on the theme of high-resolution imaging.*

EB: Certain molecules will absorb photons if you shine light on them. When the molecule absorbs a photon, it gets excited to a higher energy state. A few nanoseconds later, the molecule trickles down to a lower energy state and then returns to the ground state. As it does this, it emits a slightly redder color than what it absorbed (Fig. 1). In this way, you can spectrally distinguish the molecule. The beauty of fluorescence is that you can tag any cellular protein you want with a fluorophore. Before fluorescent labeling techniques, you were basically limited to visualizing cells as ghostly-looking bags that contained some bumps. Maybe you could distinguish a mitochondrion if you looked in an electron microscope, but you couldn't really know where the proteins were, and it's the proteins that drive what happens in the cell. Another advantage is that fluorescence only lights up the molecule you want to see, so you have a black background. This is particularly important at the single-molecule level.

BSJ: What makes an optimal fluorescent label?

EB: There is no such thing. The disadvantage to fluorescent labels is that they are not intrinsic to proteins. Instead, they are like a bowling ball that you stick on the side of a protein. One of the unmet holy grails is to get protein-specific contrast without having to attach a large, non-native molecule to the protein. No one knows how to do that, but that is one nut we would like to crack someday.

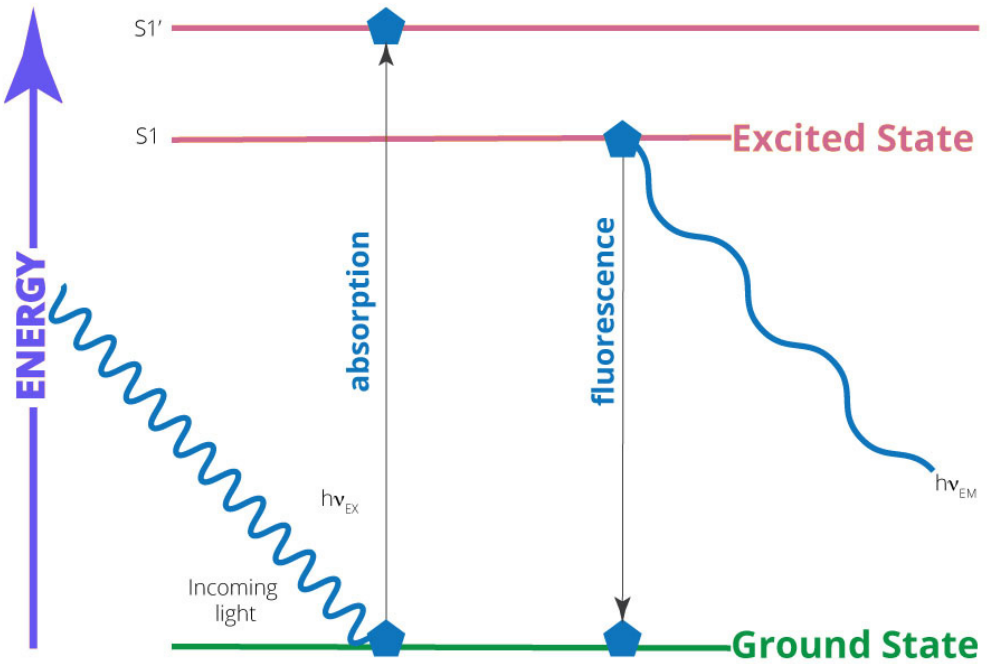

Figure 1: Electronic state diagram illustrating the principle of fluorescence.[2] *A fluorophore absorbs a photon, which causes an electron to move to a higher energy state (ground state to S1'). The electron relaxes to a lower excited state (S1' to S1), and the fluorophore subsequently emits a photon as the electron returns to the ground state (S1 to ground state). The camera detects this emitted photon as the fluorescence signal, which has a longer wavelength than the incoming photon.*

BSJ: What is the Nyquist criterion and how does it pose a problem for labeling density?

EB: If you remember the pre-HDTV era, you know how crappy the pictures were on your TV screen. The problem is that old TVs didn't have as many pixels, so the images looked coarser. The more pixels you have, the higher the resolution. The Nyquist criterion says that if I want to see something of a given size, I need to sample at least half the size of that object. This corresponds to the size of pixels in my image (Fig. 2a). If you have lots of molecules in the sample, but only 0.1% of the molecules have fluorophores on them, you don't see a continuous image of the structure. Instead, you see a random field of dots (Fig. 2b). Getting enough fluorophores on your molecule is a huge problem in super-resolution imaging. The more fluorophores you add, the more you perturb the system, but if you don't add enough fluorophores, you can't see what you want to see. So you're always playing this trade-off.

BSJ: What are some challenges associated with non-invasive live-cell imaging?

EB: Lots of challenges! The first is introducing labels in a way that doesn't perturb the physiology of the sample. Before advances in CRISPR-Cas9 genome editing, you had to add the DNA sequence of your fluorescent protein into the cell. Typically, this makes the cell produce much greater than native amounts of the protein, which makes the cell sick. Now, you can precisely edit the genome to get an endogenous level of tagged protein, which is more physiologically relevant. The biggest problem in non-invasive imaging is that the amount of light that most microscopes put on the sample is equivalent to putting you on the planet Mercury—you're not going to be happy for very long. A lot of my work post-super-resolution was about developing new microscopes that are gentler to cells so that we can look at them for longer periods of time. Finally, many biologists have been constrained to looking at immortalized cells on cover slips. Those cells are really pathological, like the HeLa cell—that cell is such a beast. It has so many extra chromosomes and it is such an anomaly, but researchers use it as a basis of studying mammalian cells. Plus, it's in isolation. In biology, the phenotypes you see are a result of gene expression, and gene expression is controlled by the environment. So if you put a cell in a non-native environment like a cover slip, it can be like the desert to the cell, even with media around it. If you don't study cells in the organism in which they evolved, how can you trust what you're seeing? A big part of our effort has been developing adaptive optics microscopes that allow us to look at cells in a more physiological context.

> "If you don't study cells in the organism in which they evolved, how can you trust what you're seeing?"

BSJ: We read about your development of photoactivated localization microscopy (PALM), which achieves nanometer spatial resolution and led to your award of the Nobel Prize in Chemistry in 2014.[4] Can you briefly explain how PALM works?

EB: The idea behind PALM is a simple one. In a normal optical microscope, you can see a single fluorescent molecule, but it's a big, fuzzy blob. You can point to its center with high pre-

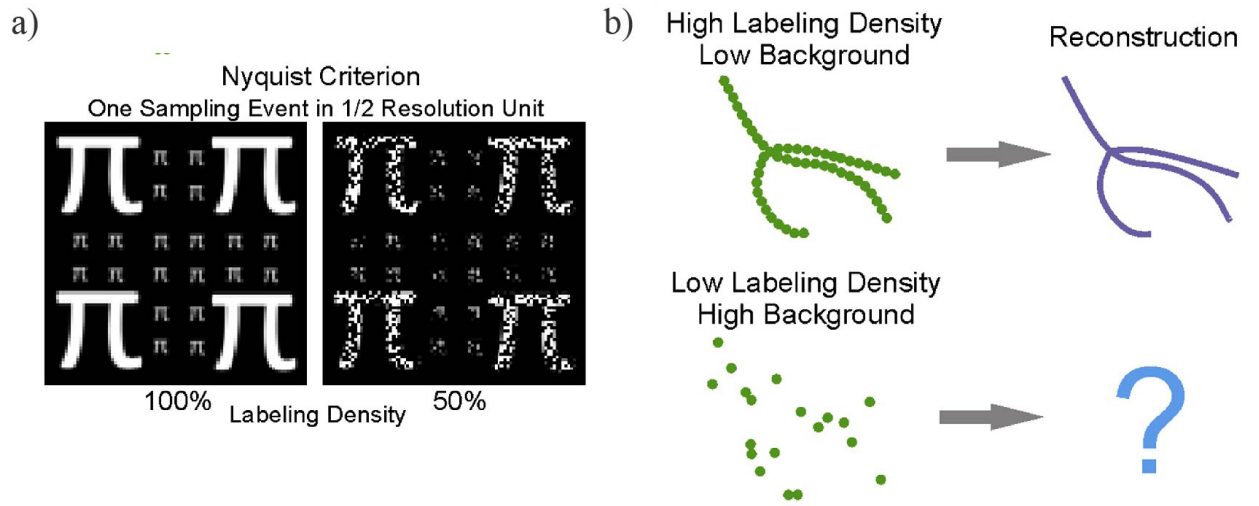

Figure 2: Importance of labeling density for achieving a clear image.[3] *(a) According to the Nyquist criterion, in order to resolve a structure (pi shape) of a particular size, the mean separation between fluorescent labels must be no greater than half of the size of the structure (labeling density must be above 50%). (b) If the structure of interest is labeled too sparsely, then its shape cannot be visualized.*

cision, but the fuzzy blobs overlap so much that you can't make any sense of them. In the early 2000s, a new type of fluorescent protein called photoactivatable green fluorescent protein was developed by my friend Jennifer Lippincott-Schwartz at the NIH. If you use photoactivation, then only a few of the fluorescent proteins light up. You can find their centers, turn off that subset, turn on another subset, and so on (Fig. 3). When my friend Harold Hess and I got that idea, we were unemployed, but it was a simple enough idea that we could do it ourselves.

BSJ: What are some current applications of PALM?

EB: PALM is used as a structural tool to complement other methods. A big area that recently got the Nobel Prize is cryo-electron microscopy, which allows you to determine the structures of individual proteins with high precision. But there can be a lot of ambiguity in how different proteins assemble into larger structures. PALM removes that ambiguity and allows you to understand exactly how things are organized in a macromolecular assembly. I'd say the most important application is in single-particle tracking PALM (sptPALM), where you photoactivate subsets of molecules and watch how they move. Our conception of how live cells work is currently undergoing a revolution. We are realizing that everything in the cell has multiple purposes and is interacting all the time. There is a "stickiness" to how different molecules come together for a tiny period of time, sometimes just hundreds of milliseconds. This is how practically every basic biochemical process works in the cell. In order to really understand the kinetics of these processes, you have to study them at the single-molecule level. For this reason, I believe sptPALM is going to become a very important tool for understanding the "glue" that holds the cell together.

BSJ: We also read about your recent study combining expansion microscopy (ExM) with lattice lightsheet microscopy (LLSM) to image subcellular structures in the mouse and Drosophila brains.[6] What are ExM and LLSM and why did you choose to combine them for imaging brain tissue?

EB: Expansion microscopy came out of Ed Boyden's group at MIT.[7] A tissue is infused with a polymer gel, and fluorophores are chemically linked to the gel. Then you add chemicals that digest all of the biological tissue. You change the osmotic balance of the solution, and the gel expands, giving you a sample that is physically four times bigger than it was before. With super-resolution microscopy, you only focus on one part of one cell—it's like imaging while looking through a straw. You get a really good view of one region, but you have no idea what's happening all around it. Expansion microscopy allows you to see over a much wider field of view. The disadvantage is that as you image sections of tissue, the surrounding fluorophores burn out. Instead of bringing the light from above, lattice light sheet microscopy allows you to bring the light from the side. It also uses ideas derived from Bessel beams, which allow you to make a narrow sheet of light that illuminates only a thin slice of sample at a time, so you don't bleach the fluorescence from regions above and below the layer. The LLSM is very fast because it illuminates an entire plane at once. We can easily acquire

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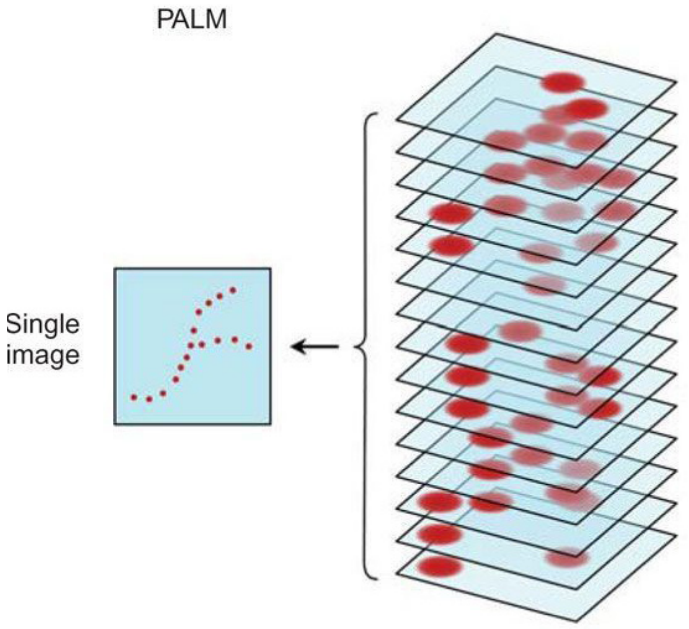

Figure 3: Principle of photoactivated localization microscopy (PALM).[5] *Photoactivatable fluorophores are attached to a protein of interest. Sparse subsets of fluorophores are repeatedly activated, imaged, and bleached. The position of each fluorophore is precisely determined in each frame. Summing together all the frames results in a single super-resolution image of the protein distribution in a sample.*

terabytes of data per day. The normal fly brain is already gigantic on the super-resolution scale, but we were able to image it in just a couple of days. Electron microscopy took about 10 years to accomplish the same task—that's the kind of gain you get with ExLLSM.

BSJ: Why was the *Drosophila* brain an ideal sample for ExLLSM?

EB: First, the study was conducted at Janelia Research Campus, where half of the building studies the fly brain. It is probably one of the most studied systems in neuroscience. Researchers at Janelia have developed fluorescence tools to study the fly brain; they have over ten thousand different genetically perturbed flies. Another nice thing is the scale. While it is certainly a lot bigger than other systems, the fly brain is still tiny enough to fit in our microscope without us having to carve up the brain into many different pieces.

BSJ: In this study, you calculated the brain-wide distribution of synapses in the fly (Fig. 4). How did you validate that the signals accurately represented true synapses and not non-specific background?

EB: That's a good question. In this case, we used fluorescent antibodies, which don't always go to the protein that you want. Electron microscopy (EM) researchers have been working on the fly brain for a long time, and one of the areas of the brain that they have studied exhaustively is the mushroom body, where a lot of learning and memory occurs. By EM, they were able to determine the number and sizes of the synapses. In our study, we developed a pipeline to count the synapses, and we removed any fluorescence signal that was below or above the size of a typical synapse. We then compared our data to the distribution of synapses that the EM researchers found. Ours was a two-color experiment, where we also labeled the dopaminergic neurons across the brain. Some dopaminergic neurons don't have any synapses on them, which has been known from EM. So, you can determine the rate of false positive signals by seeing if there are any synapses on a neuron that shouldn't have synapses. We counted no synapses on the neurons that shouldn't have any and tens of thousands of synapses on the neurons that should. Everything matched up perfectly in all of these controls.

BSJ: How might ExLLSM allow for the correlation of neural structure with neural activity?

EB: There are two ways that people look at neural activity. The gold standard is to use electrodes to determine the electrical signals coming from neurons. That's great, but it's difficult to stick a needle into each neuron. A big area of the last 15 years has been genetically encoded calcium indicators. You can express a calcium-sensitive fluorescent protein in any subset of neurons you want. When an action potential is fired in a neuron, there's an influx of calcium and the neuron lights up for the amount of time that it's firing. This calcium influx is optically read out. At Janelia, researchers have done experiments in which a fly looks at a virtual reality screen and has to follow prey. There are little microscopes that look at the neurons as they fire. You can then kill the fly, take out its brain, and do ExLLSM. This way, you can relate the neural activity to the neural structures and behavior of the fly.

BSJ: You have experience in industry and academia, and you have worked as both an engineer and a scientist. What do you think are the most valuable elements of this skill set?

EB: Several things. First, regardless of what you do, be true to yourself and listen to your internal voice. There's a saying that 90% of everything is BS—that's totally true. You have to be your own toughest critic. There are a lot of things one can do to cut corners or go with the crowd, even if you don't believe what the crowd is saying. But I think there's a larger price to pay in the long term if you do that. Be focused on what you believe is important. The other thing is the value of hard work, which is universal regardless of what you do. I've known many brilliant people in my life, but the people who have succeeded in the end are the ones who are driven, passionate, and work, work, work. If you work 20% harder, that added

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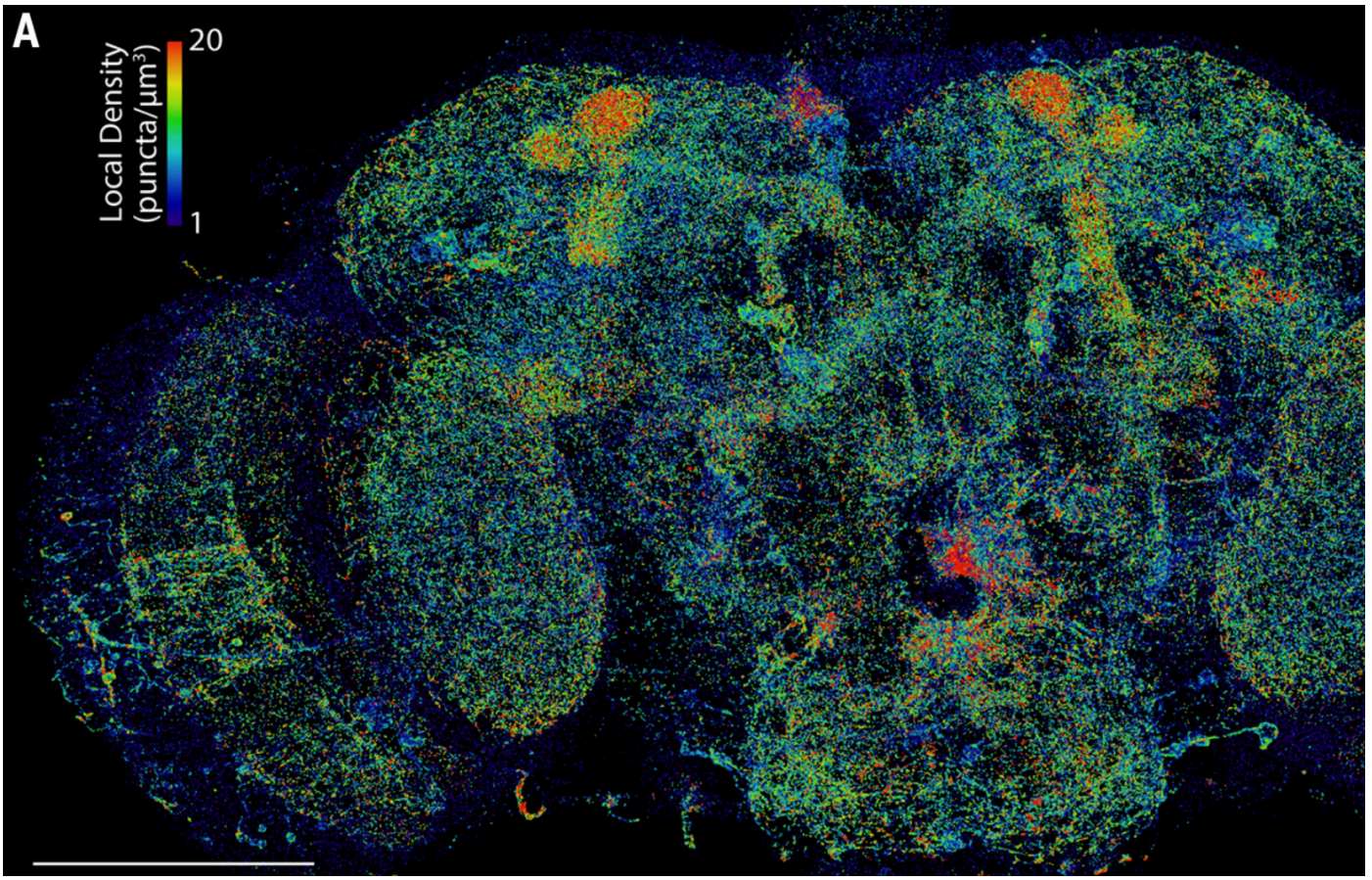

Figure 4: Maximum intensity projection of synaptic proteins. *Synaptic proteins at dopaminergic neurons in the adult Drosophila brain, color-coded by local protein density.*[6]

20% gives you more experience and makes you more productive every year. It really compounds over time until you're two or three times as productive as the next guy. That's not easy, because in life you always have to make compromises of family, work, sanity, and health. But still, the hard work is what will make you stand out and have a real contribution in the end. Be true to yourself and work hard—that's true whether you're in academia or industry.

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