Single Molecules, Cells, and Super-Resolution Optics

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One of the nicest things about winning the Nobel Prize is hearing from all of the people in my past and having the time to reflect on the important role they've each played in getting me to the happy and fulfilling life I have now. To all of my friends and colleagues from grade school up to my peers who nominated me for this honor, you have my deepest thanks.

I was first introduced to super-resolution back in 1982 when I went to Cornell University for graduate school and met my eventual thesis advisors, Mike Isaacson and Aaron Lewis. Mike had recently developed a means of using electron beams to fabricate holes as small as 30 nanometers (Figure 1A). He and Aaron figured that if we could shine light on one side of the screen, then the light that initially comes out of the hole from the other side would create a sub-wavelength light source that we could then scan point-by-point over a sample and generate a super-resolution image [1] (Figure 1B). The ultimate goal was to try to create an optical microscope that could look at living cells with the resolution of an electron microscope. I wanted to become a scientist to do big, impactful things, and that certainly fit the bill, so I said, "Please sign me up."

At the time, many people told us that this idea would never work, either because it violated Abbé's law, or even worse, the Uncertainty Principle. I didn't find their arguments compelling, but all doubt was removed from my mind in 1984 when we learned about the work of Ash and Nicholls. In 1972, they used

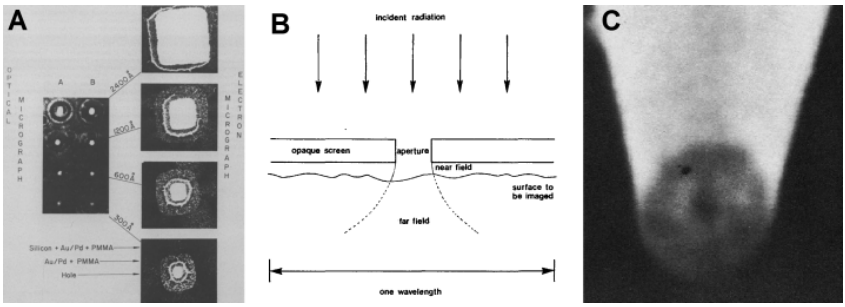

FIGURE 1. Apertures and near-field optics. (**A**) Electron beam-fabricated apertures in a silicon nitride membrane, with diameters as shown. Column at left shows light transmission through these apertures [1]. (**B**) The concept of near-field scanning optical microscopy (NSOM) as a path to super-resolution [2]. (**C**) 50 nm aperture in a tapered glass pipette coated with opaque aluminum [3], made using techniques developed for patch clamp recording [4].

3-centimeter microwaves and were able, by near-field techniques, to get resolution of 1/60 of the wavelength λ in test patterns in a beautiful paper in *Nature* [5] (Figure 2). In fact, the idea for near-field microscopy goes back even further, to E.H. Synge in 1928 [6], and many people have independently come up with the idea since.

The first far field demonstration of breaking the Abbe limit of $\lambda/(2NA)$ (NA being the numerical aperture of the objective) in the far field goes back even further, to the work of Lukosz [7]. By introducing grating masks at planes conjugate to the object and the image, he was able to image test patterns at three times Abbe's limit [8] (Figure 3A). This was the forerunner of what is known today as structured illumination microscopy. Lukosz' demonstration was at very

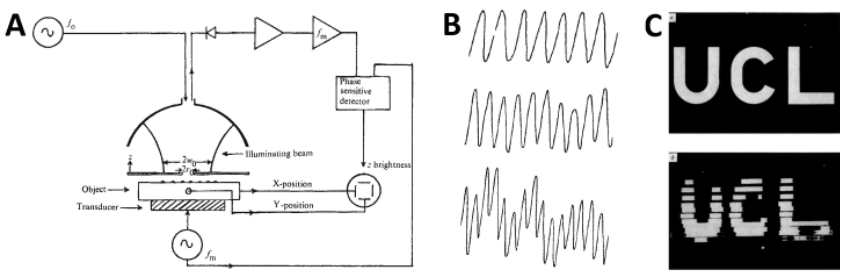

FIGURE 2. Breaking the diffraction barrier in the near field. (**A**) Microwave resonator with sub-wavelength aperture as used by Ash and Nichols in 1972 [5]. (**B**) Resolved gratings having periods of 1/30, 1/40, and 1/60 (top to bottom) of the 3 cm microwave wavelength. (**C**) Images of letters having linewidths of 1/15 of the wavelength.

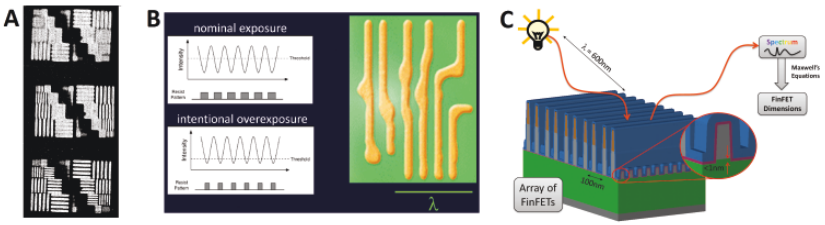

FIGURE 3. Breaking the diffraction barrier in the far field. (**A**) Diffraction-limited (top) and super-resolved test patterns in 1D (middle) and 2D (bottom) as seen by Lukosz in 1967, using linear grating or square grid masks inserted in the image path [8]. Resolution is increased to three times the Abbé limit. (**B**) Exploiting nonlinearity in photoresist development and double patterning to create features beyond the Abbé limit during the production of integrated circuits [9]. (**C**) Exploiting the *a priori* knowledge of a desired circuit pattern, and comparing the distribution of scattered light from the actual pattern to the predicted distribution, to measure features sub-nm features to sub-Å precision in high volume semiconductor manufacturing [9].

low NA, so the features were still much larger than the wavelength of light, but it nevertheless demonstrated that Abbé's law was not inviolate.

In fact, far-field super-resolution has a very long history, particularly in the semiconductor business, where the nonlinear interaction of light with photoresist has been a staple of making linewidths far smaller than the Abbé for a generation (Figure 3B) [9]. Even more impressive, though, is how visible light is used to inspect semiconductor wafers, and how by having *a priori* knowledge of the pattern you hope to create, developing a model for the diffraction of light from that pattern, and comparing the actual data you get against the model, people today are able to measure features in the pattern down to about 1/1000 of the wavelength of light (Figure 3C) [9]. This is used day in and day out in high volume semiconductor manufacturing.

So really, at some level, super-resolution is not new at all, and there are people in Silicon Valley who are probably laughing at us here today for thinking that we're the guys who invented this, when it has been a staple for such a very long time. Nevetheless, in my mind Ash and Nichols deserve the lion's share of the credit for being the first to not just push slightly beyond the NA-dependent Abbé limit of $\lambda/(2\text{NA})$, but to shatter the diffraction barrier completely by going way beyond the seemingly more fundamental limit of seeing beyond half the wavelength of light, and getting to $\lambda/60$ with the near-field technique.

Speaking of shattering, the types of apertures we were making in those thin membranes would break all the time; they were hard and time-consuming to make, and they were costly. So eventually we abandoned that and, using an idea

from my fellow grad student Alec Harootunian, we instead pulled glass micropipettes, similar to the method that was developed just a few years before in patch clamping for single ion channel recording. We would then coat these with aluminum to create an opaque structure, except for the little hole at the end that would then be our aperture [3] (Figure 1C).

So with that, I built the monstrosity you see here (Figure 4A), which was my first near-field optical microscope. I cringe now at how complex and crazy this thing was, but at least it gave me the ability to learn the system-engineering skills I would need to become a true engineering physicist, and eventually I was able to surpass the diffraction limit [10] (Figures 4B,C [11]) with this microscope that I built for my Ph.D. thesis.

That microscope was frankly a pain in the ass to work with, and reliably the resolution gain was about a factor of two beyond Abbé's limit. But it was good enough to get me my dream job at Bell Labs. I started trying to develop the technique further and, for the first two years, progress was really slow. But thanks to the patience and encouragement of my boss, Horst Störmer, I eventually came to realize that that pipette probe was not a really good design, because the light that was sent down the taper was largely retro-reflected back before it ever got to the tip, and the little bit of light that did make it to the tip was in electromagnetic modes that didn't couple well to the aperture.

Postdoc Jay Trautman and I, then, instead created a probe that consisted of an adiabatically-tapered optical fiber, which would guide the light very efficiently to the tip region, and then efficiently couple that light to the evanescent modes in the aperture (Figure 5A,B). This made a probe that was 10,000 times brighter than the earlier ones, and also then allowed us to routinely get to about 50-nanometer resolution [12] (Figure 5C). In the following year I also invented a means to dither the probe back and forth sideways—oscillate it—and then as it would come close the surface, the oscillation would be damped. By that, I could regulate the distance of the tip from the sample [13] (Figure 5D).

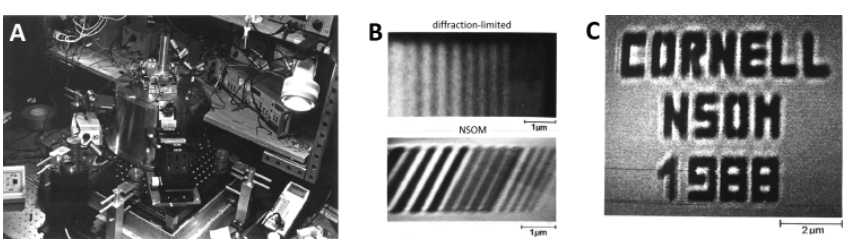

FIGURE 4. My first near-field microscope. (**A**) The microscope itself. (**B**) Test-pattern comparison of diffraction-limited imaging versus super-resolved NSOM. (**C**) Another resolved test pattern, i.e., an early lesson in learning how to sell my work [11].

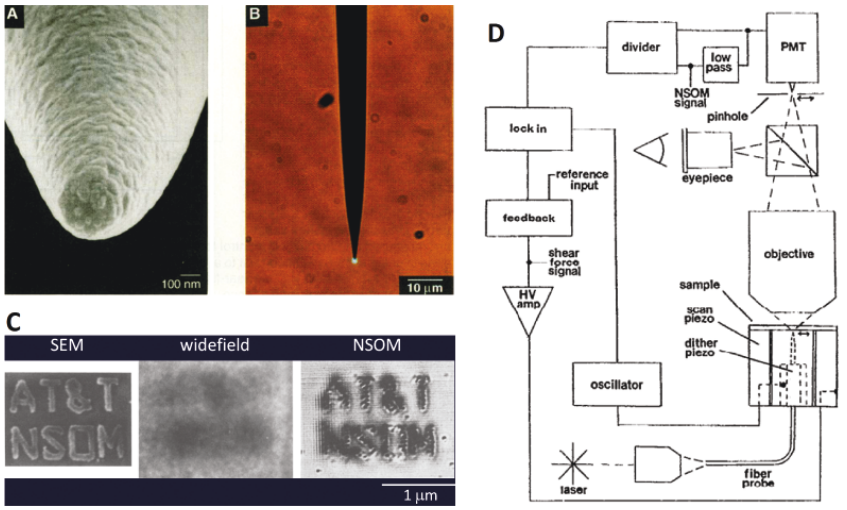

FIGURE 5. Making NSOM a real tool. (**A**) Electron micrograph and (**B**) Optical micrograph of an adiabatically tapered, aluminum coated single mode optical fiber used as a near-field probe [14]. (**C**) Resolution comparisons with the probe. Left to right: electron, conventional optical, and near-field optical micrographs [12]. (**D**) Schematic diagram of shear force feedback for regulating, at the nm scale, the distance from the aperture at the end of the probe to the specimen [13].

With these two innovations, near-field became fairly routine. In 1992, we had the world record for data-storage density, when we could read and write bits as small as 60 nanometers in a magneto-optic material [15] (Figure 6A). We also demonstrated super-resolution photolithography (Figure 6B) and nanoscale spectroscopy [16], and exploited various contrast mechanisms [14], including

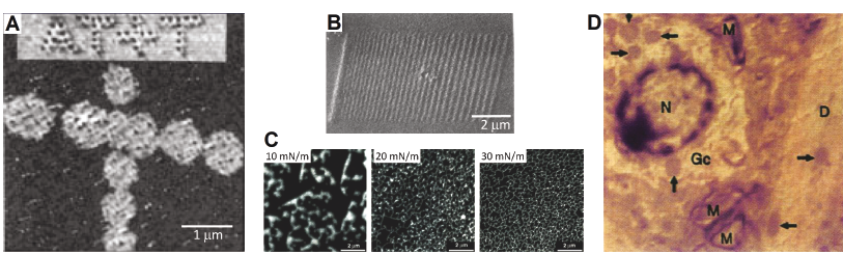

FIGURE 6. The golden age of NSOM. (**A**) Single bits of information (top) in a magneto-optic film recorded and read out by NSOM, compared with bits (white) recorded with conventional optics [15]. (**B**) Near-field photolithography [14]. (**C**) Fluorescence imaging of phase transitions in phospholipid monolayers [17]. (**D**) Histological stained section from the monkey hippocampus [14].

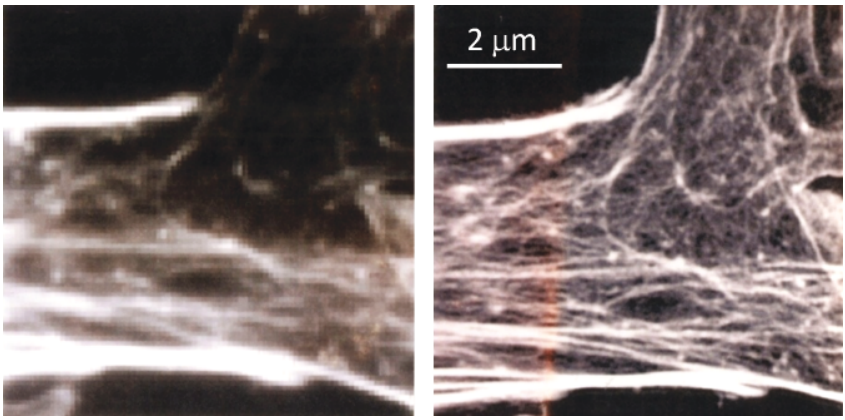

FIGURE 7. Super-resolution fluorescence imaging of cells. Conventional widefield (left) and super-resolved NSOM (right) images of cytoskeletal actin in the same region of a fixed mouse fibroblast cell [18].

refractive index, absorption (Figure 6C), polarization, and fluorescence [17] contrast (Figure 6D). In fact, to this day, near-field remains the only diffraction-unlimited technique which can use the full panoply of optical contrast mechanisms and isn't dependent on a switching mechanism in fluorescence.

Nevertheless, the mechanism that's probably most important for biology is fluorescence, because it offers protein-specific contrast. In 1993, we were the first to demonstrate super-resolution fluorescence imaging of cells when we looked at the actin cytoskeleton in the flat lamellar region of fixed fibroblasts [18] (Figure 7). What was particularly exciting about this, though, was that the signal-to-noise ratio we achieved on these single actin filaments, coupled with our knowledge of the aperture diameter, suggested that it should be possible to image single fluorescent molecules. This was a very hot topic at the time, because just a few years previously W.E. Moerner [19] and Michel Orrit [20] had broken to this ultimate level of sensitivity at cryogenic temperatures, and several groups, such as those of Dick Keller [21] and Rudolf Rigler [22], had already shown at room temperature in solution that you could see bursts of fluorescence from single molecules.

The key to these later experiments was the idea that you had to restrict the excitation volume to reduce the background. That's what near-field excels at—confining the excitation volume. As soon as Rob Chichester and I decided to try to look at single molecules, on our very first try we got really great results. But the weird thing was, instead of seeing a bunch of round spots, they would instead look like these crazy arcs or ellipses or other things, and these would

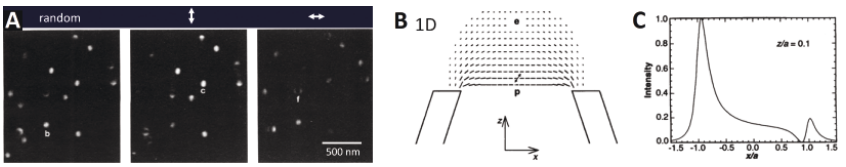

FIGURE 8. Single molecule microscopy at room temperature. (**A**) Three views of the same field of carbocyanine dye molecules on PMMA as imaged by NSOM with three different polarizations as shown at top. (**B**) 1D schematic of the interaction of the electric field **e** emerging from the near-field aperture with the electric dipole moment **p** of a single molecule. (**C**) Resulting intensity $I(x) \propto |\mathbf{e}(x) \cdot \mathbf{p}|^2$ recorded as the aperture is scanned across the molecule [23].

change as we changed the polarization of the light [23] (Figure 8A). I still remember running excitedly to Horst's office and trying to understand this, and together with his help, realizing that what we were seeing was the interaction of the electric dipole moment of the molecule with the evanescent fields inside of the near-field aperture (Figure 8B,C). And that was what was giving rise to these patterns.

And so, what that means is that we could turn the experiment around and think of the molecule as the light source and the aperture as the sample. By choosing molecules that were oriented along the *x*, *y*, and *z* axes, we could then map out the nanoscopic electric fields inside the aperture (Figure 9A, center column). We then compared this to a theory for near-field diffraction that Hans Bethe had developed back in 1944 [24] and were able to show very good agreement (Figure 9A, other columns). Once we had that, then we could use Bethe's model to predict what kind of pattern we would see for any orientation of molecule, compare that to our data, and hence find the dipole orientation (Figure 9C)

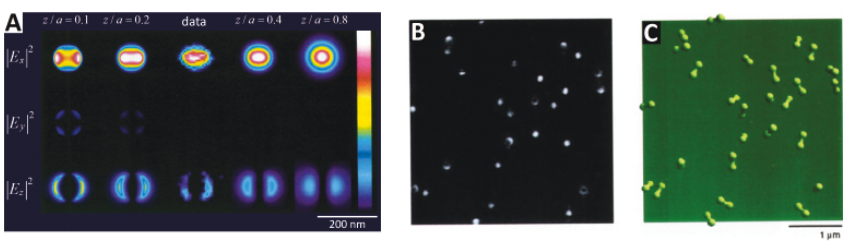

FIGURE 9. Mapping nanometric electric fields and measuring single dipole orientations. (**A**) Electric field components (rows) predicted near a sub-wavelength sized aperture at different distances (columns) from the aperture, compared to experimental components (center column) measured with single molecules. (**B, C**) Orientations of single molecules determined by matching measured to predicted emission patterns [23].

of every molecule in the field of view (Figure 9B). And given that information, we were then able to fit these crazy shapes to the theory and find the positions of these molecules down to about 12 nanometers in x and y, and about 6 nanometers in z. This became very influential for what was to happen later.

In another pivotal experiment, I joined forces with my best friend and colleague at Bell, Harald Hess. Harald had made a name for himself at Bell a few years earlier by building a world-class cryogenic scanning tunneling microscope with which, among other things, he discovered the core states at the centers of the vortices in the Abrikosov flux lattice of type-II superconductors. Harald's and my interest was to combine my near-field probe with his low-temperature scope to be able to study excitons, which are the sources of light generation in semiconductor heterostructures, such as in this laser pointer, that won the Nobel Prize in 2000. Our goal was to combine the high spatial resolution obtainable with my near-field probes with the high spectral resolution we could get in Harald's rig by running near absolute zero (Figure 10A).

When we did this [25], we were surprised to find that the normally smooth spectrum that you see instead would break up into these crazy sharp lines. And furthermore, as we drove the probe even small distances from point to point, this spectrum would change completely (Figure 10B). What we eventually realized is that we were seeing that the excitons could not emit anywhere, but were confined to only certain specific points of exciton recombination, and the color of the light emitted at one of these points was based on the local thickness of the quantum well at that point. What was probably more important later on was that even though there might be a dozen or more of these emitting sites underneath our tiny near-field probe, we could still study them individually because they glowed in different wavelengths. So if we built up this higher dimensional

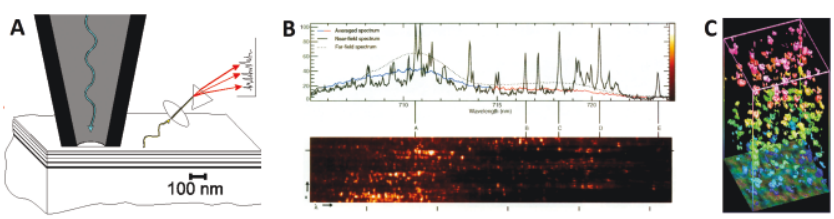

FIGURE 10. Near-field cryogenic spectroscopy of quantum wells. (**A**) Experimental schematic, showing a near-field probe (left) exciting a multiple quantum well structure (bottom), with the resulting emission measured at a spectrometer (right). (**B**) Comparative near-field and far-field spectra at a single point (top), and spectral changes with position (bottom). (**C**) Images of emission from single exciton recombination sites isolated form one another in a 3D space of position x,y and wavelength λ [25].

space of *x*, *y* and emission wavelength, we could study them individually (Figure 10C).

By this time in 1994, the limitations of the near-field technique were incredibly obvious. Some of these were just engineering challenges, but some were truly fundamental. The foremost of these is that the near-field is ridiculously short. It was clear that, because of this short depth of focus, there was no way I was going to realize my ultimate dream of looking at live cells with the resolution of an electron microscope, so I got very frustrated. At the same time, though, near-field got to be a big fad, and like all scientific fads, you get a lot of people jumping into the field. They publish sloppy results, sweep all the problems underneath the rug, and over-hype the capabilities. All of that made me very uncomfortable. And the third thing that tipped the balance for me was Bell. You had to work really hard to succeed at Bell, but by 1994 you could sense the changes that were happening in the company, and they would no longer value basic science in the way they used to. All these things together took two young and innocent guys like me and Harald in 1989 and turned us into two stressed and worn-out guys just five years later.

So with all of that combined, I said, "Screw it, I'm sick of science. I really hate academia. I quit." So that's just what I did. I really had no idea what I was going to do next. But after a few months of trying to flush near-field microscopy out of my head, I was walking my daughter around in a stroller and it hit me—I don't know how or where from—that you could combine that single-molecule

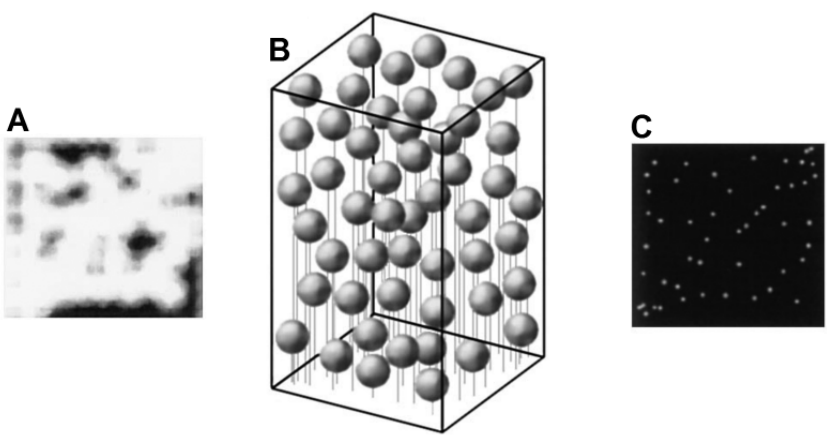

FIGURE 11. The concept of super-resolution localization microscopy. (**A**) A field of close-packed molecules unresolved, because their diffraction-limited images overlap. (**B**) The same molecules, after their mutual isolation in a higher dimensional space. (**C**) Super-resolution map of molecular positions after localization of each isolated molecule [26].

experiment I did with the spectroscopy experiment Harald and I did to come up with a different far-field way of doing super-resolution imaging.

The idea is that if you have a bunch of molecules that are too close together, their diffraction-limited spots overlap (Figure 11A). We've already heard about this in W.E.'s talk. However, if you can find some way in which they differ from one another—and it can be anything—then you can isolate them in a higher dimensional space (Figure 11B). But once they're isolated, you can find the centers of each one of their diffraction-limited spots to much better than the width of the spot, and hence, you plot all the coordinates of the molecules (Figure 11C).

I published that idea in 1995 [26]. In 1999, van Oijen and colleagues first demonstrated this by spectral isolation at low temperature and resolved seven molecules in one diffraction-limited volume in 3-D [27]. In the 2000s, several groups extended this to room temperature, by various means—photobleaching [28,29], lifetime [30], or blinking [31]. This was really a general concept I was trying to get across here.

The problem with all of these methods, though, is based on something called the Nyquist criterion (Figure 12). If you want to make any microscope image of

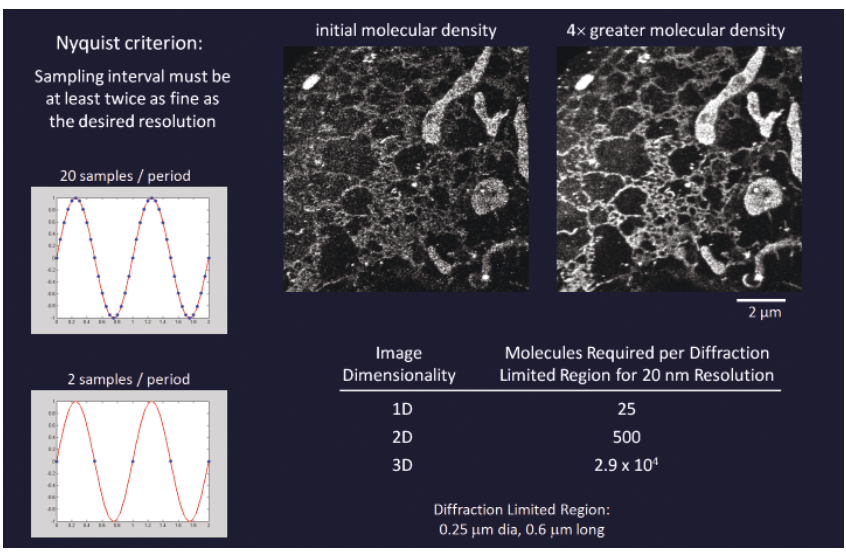

FIGURE 12. The Nyquist criterion and the labeling density problem. With many localized molecules per period, any spatial frequency is easily detectable (center left), but when the number drops to two per period or less, the frequency might be missed. As a result, resolution in localization microscopy is limited more by density of single molecule labels than by the precision their localization (top right). The minimum number of localizations required per diffraction-limited region increasing rapidly with dimensionality (lower right).

a certain resolution, you have to sample every resolution element divided by two. For example, if I sample only once every half period of this sine pattern (Figure 12), I can miss it completely (Figure 12, lower left). What that means is that if you want to get 20 nm resolution in two dimensions by this method, you have to have the ability to see one molecule on top of 500 that could be glowing at the same time. And none of the methods I just described were at the point of having that much isolation in that third dimension to get very much beyond the diffraction limit. I didn't have a really good idea in 1995 about how to get around this problem, other than running at cryogenic temperatures with a near-field microscope. That was going to be a hero experiment, and I was sick of science, so I just published the idea and left it at that.

Eventually I ended up working for my Dad's machine tool company in Michigan, where I did a number of things, but my baby, the one I'm proudest of, was a servo-hydraulic machine tool that married old hydraulic technology to modern control algorithms and the sort of energy storage principles that you find in hybrid cars today. It would move 4 tons at 8gs of acceleration and position it to 5 microns, while collapsing the size of the machine from the size of this stage to something about the size of a car, making it much cheaper, much faster, much more productive. I spent four years developing that idea, and three years trying to sell it, and in the end I sold two. So what I learned is that I may be a bad scientist, but man, am I a worse businessman.

By 2002, I said, "Dad, I'm tired of wasting your money. You know, I'm sorry, this just isn't going to work." And so I quit. As usual, I had no plan B. This was the darkest time in my life, because not only had I pissed away my academic career, I had also blown up my backup plan of following in my Dad's footsteps. I'm 42 years old with two young kids and no job and no prospect of a job.

But I did something smart. Harald had also gone into industry, where he was considerably more successful than I was, working for a startup in San Diego. So I reconnected with him and we just started getting together in different parts of the country, like the national parks, and just talked . . . What's the meaning of life? How can we have an impact before we die? What's interesting? What we realized is that while neither one of us fits well in the normal academic scheme of things, we both really love science and we love the ability to be able to pursue our curiosity. So we started trying to think about what we could do to have an impact in science again.

That caused me to start reading the scientific literature, which I hadn't done for 10 years. The first thing I ran across was green fluorescent protein [32]. It was a revelation to me—a shock—because it was such a big problem in the near-field days, how to label cells to get high enough labeling density and specificity. The

notion that you could coax a live cell with a little bit of jellyfish DNA to be able to get it to produce any protein you want with a fluorescent tag on it. My jaw was hanging down for a week in astonishment at this. Initially when I was casting around for an idea, I didn't want to do microscopy, but as soon as I saw this I said, "Oh, shit, I've got to do microscopy again."

While Harald and I continued to look around during my holiday from science, science itself wasn't standing still. Right after GFP appeared on the scene, a lot of people wanted to understand its photophysics, in part to be able to do mutagenesis to get different colors so they could do multicolor imaging.

Steven Boxer's group in 1996 noticed that there isn't just one absorption hump for GFP but two. And what was even crazier is that if you would excite at this near-UV peak for a while, it would go down, but the peak at 488 would go up [33] (Figure 13A). In other words, there was some kind of weird photoactivation effect happening in GFP.

Then Tobias Meyer's group actually exploited this for what was the first photo-activated pulse-chase experiment, where they used wild-type GFP and focused UV light to enhance the brightness of GFP in a certain part of the cell, and then watched how those proteins go to other parts of the cell [34] (Figure 13B). The following year, W.E. was able to show the same phenomenon with Rob Dickson in GFP at the single molecule level [35] (Figure 13C). Then, around 2000, George Patterson in Jennifer Lippincott-Schwartz's group at NIH was very

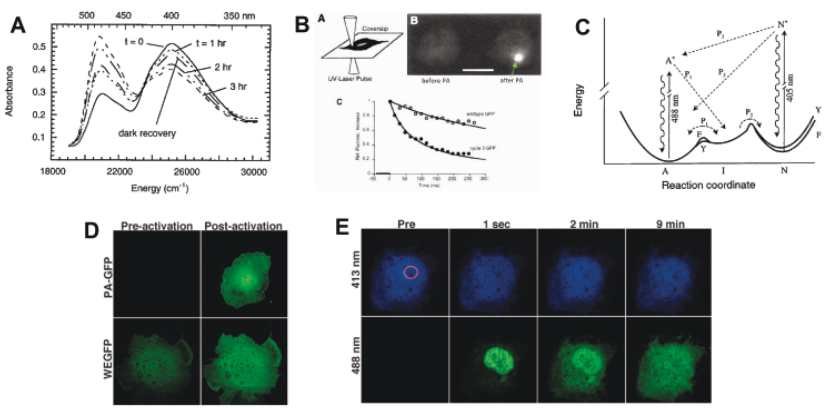

FIGURE 13. The development of photoactivatable GFP. (**A**) UV-visible double absorption peaks of wt-GFP [33]. (**B**) Pulse chase experiment to trace the fate of wt-GFP locally photoactivated in one region of a cell [34]. (**C**) Energy state diagram from single molecule photoactivation of GFP [35]. (**D**) Improved on/off contrast ratio of PA-GFP [36]. (**E**) Pulse chase experiment of the relative rates of nuclear and cytosolic diffusion of PA-GFP [36].

interested in following up on what Tobias had done. The problem was that the on/off contrast ratio for wild-type GFP was very low, so he applied directed mutagenesis and eventually came up with what was called PA-GFP [36]. With this, you could turn on the fluorescence of molecules with a much higher contrast ratio (Figure 13D), and use them in much better pulse-chase experiments (Figure 13E).

In 2005, Harald recommended that we go visit the National High Magnetic Field Lab that was headed by our buddy from Bell, Greg Boebinger, so that we could meet some guy named Mike Davidson. Mike was a microscopist who had made a fortune selling neckties that were emblazoned with photomicrographs of cocktail mixes, and he channeled that money into creating the website tutorials for the major microscope companies. He made a lot of money from that, and then used that to follow his passion of doing live-cell imaging. Eventually he developed a library of 3,500 different fluorescent protein fusions. When we visited Mike, Harald and I learned about photo-activated GFP and the other photo-activated proteins that had come along. I vividly remember Harald and I sitting in the airport in Tallahassee and then both of us being thunderstruck when we realized that this idea of being able to turn on molecules one at a time was the missing link to make that idea I had pitched 10 years earlier to work.

I had been pursuing another microscope idea at the time [37,38]. We dropped that like a hot potato. We thought, this is easy—let's do it and do it now. The problem is that Harald had quit his job a few months before. So now you have two guys who are unemployed—how the hell are we going to do this? It's going to take too long to get a grant, too long to get VC funding. So because Harald doesn't burn his bridges as effectively as I do, he was able to take a lot of

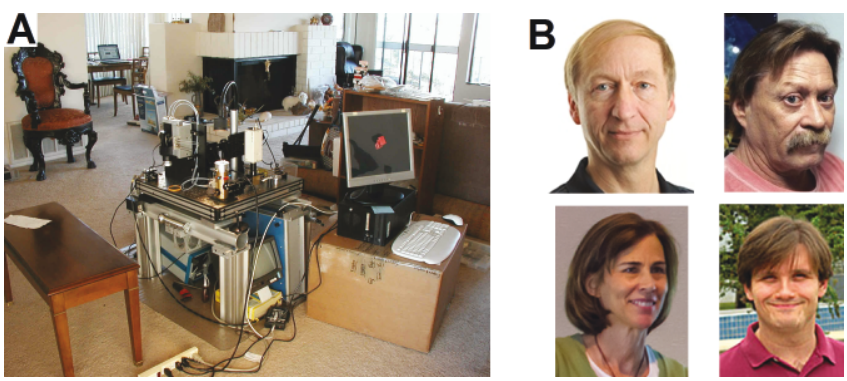

FIGURE 14. The development of PALM. (**A**) La Jolla Labs, also known as Harald's living room. (**B**) The PALM team. Clockwise from upper left: Harald Hess, Mike Davidson, George Patterson, and Jennifer Lippincott-Schwartz.

his equipment from Bell. We pulled that out of the storage shed, and put $25,000 each of our own money into it. Normally you would do it in the garage like Jobs and Wozniak, but we were able to put it together in Harald's living room (Figure 14A) because he wasn't married. So there was nobody in the way to prevent that from happening. But we knew we had to work fast because this idea was going to be ripe and in the air, so we worked around the clock day and night in order to do this—or at least Harald worked day and night. I found the couch sometimes too comfortable, so Harald would tease me and keep taking pictures of me while I was asleep.

We were still missing one thing, though, as you had two physicists who were totally naïve about biology. We needed a good partner in that regard. So, shortly after the visit to Mike Davidson, I gave a talk at NIH that I wangled after contacting another Bell Labs friend, Rob Tycho. In the talk, I pitched the other microscope idea, but I asked—when I'm there could I please, please, please meet George Patterson and Jennifer Lippincott-Schwartz?

I took George and Jennifer to lunch and I swore them to secrecy and told them the idea that Harald and I were working on. Many people would have blown us off because we were two crazy guys who hadn't published a paper in 10 years. Jennifer doesn't think that way, and I owe a lot of my success to her as well as Harald. She said, "Fantastic, bring it here." Great! Now we had the team we needed (Figure 14B). After building the instrument in Harald's living room, we packed it up and started working in the darkroom in Jennifer's lab, which was a lot less comfortable.

We started doing experiments very quickly after we brought it in. George did all the cell culture, transfections, and molecular biology to try out a whole bunch of different protein fusions. We turned down the violet light so low that a few molecules at a time would come on. If we summed up those spots, we got the diffraction-limited image. But instead, if we found the center of each spot first and then plotted those, we started building the PALM image (Figure 15A). After 20,000 frames, we went from the diffraction-limit to a super-resolution image [39] (Figure 15B). With high enough labeling density you can get down to 20 nm resolution in your living room by this technique. It's a fairly simple method.

In a way, Harald and I got lucky, because it wasn't certain that we'd be able to localize enough molecules to meet the Nyquist criterion at very high resolution. We got lucky in the sense that we found certain photo-activated proteins (Figure 16A) and caged dyes (Figure 16B) that had enormous on/off contrast ratios. There's now a lot of work in this field, and I feel many people still don't appreciate how important that on/off contrast ratio is to get from smushy looking

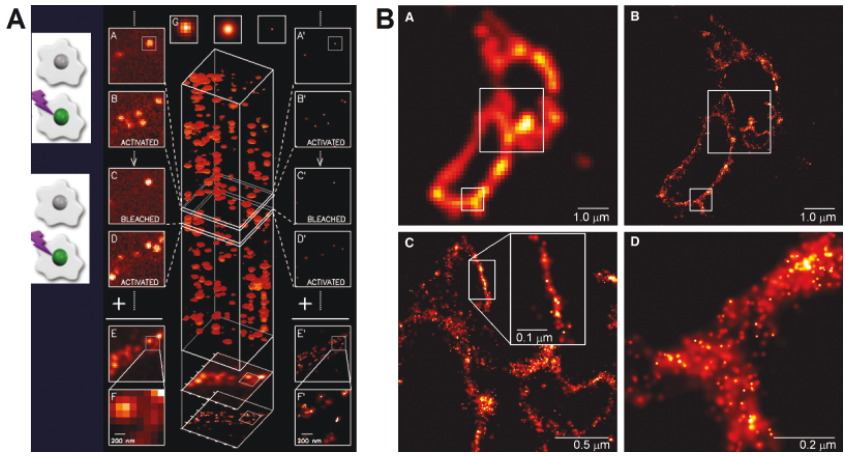

FIGURE 15. Photoactivated localization microscopy (PALM). (**A**) Repeated rounds of weak photoactivation with violet light activates different subsets of molecules in a specimen. Summing their diffraction-limited spots produces a diffraction-limited image (left column), but summing the measured centers of all such spots produces the super-resolution PALM image (right column). (**B**) Diffraction-limited (upper left) and super-resolution PALM images in different regions, showing the distribution of transmembrane protein CD-63 in a 70 nm section cut through lysosomes in a COS-7 cell [39].

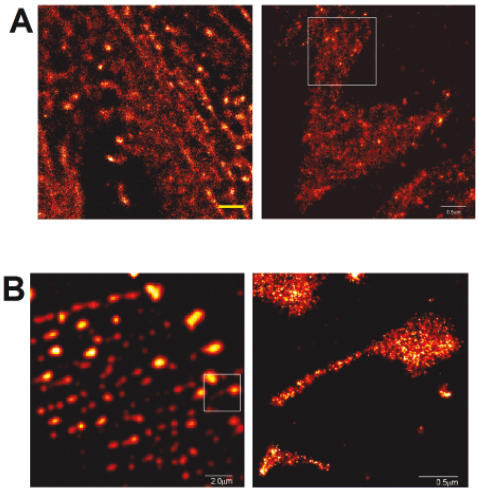

FIGURE 16. The importance of high molecular on/off contrast. (**A**) PA-GFP (left), with a poor contrast ratio, yields poor resolution in a PALM image of a focal adhesion, due to imprecise molecular localizations from surrounding background. The photoactivatable fluorescent protein Eos (right), with a high contrast ratio, achieves better resolution [40]. (**B**) Diffraction-limited (left) and higher magnification PALM images (right) of islands of high contrast caged Q-rhodamine dye [39], demonstrating that PALM is not limited to just fluorescent proteins.

results like that to much crisper results because of the background problems that you face [40].

We went from the idea of PALM to having the data for our *Science* paper that got me on this stage today in six months. That's what we could do because we were working alone in a living room, which is a very effective environment. But 2005 was the luckiest year of my life, and not only because of PALM. In the same year, by a different, crazy set of circumstances, I got introduced to Gerry Rubin. HHMI (Howard Hughes Medical Institute) was starting to build a freestanding research institution modeled on Bell Labs. The rebirth of Bell Labs caught Harald's and my interest. Gerry was farsighted enough to hire two guys who hadn't published a paper in 10 years—this was before the PALM paper came out. And so we went from rags to riches.

Once the institution (Janelia Farm) opened, we went and built the next generation scopes. I hired postdoc Hari Shroff, Harald hired Senior Scientist Gleb Shtengel, and then we went to work. In my group, we focused on applications for the first few years. With Jan Liphardt's group at Berkeley, we looked at chemotaxis receptors in *E. coli* [41] (Figure 17A), and were able to show that the various cluster sizes you see and their positions along the poles are completely predictable in terms of stochastic model of self-assembly, where the proteins are randomly inserted in the membrane and then diffuse until they stick to an existing cluster. We also showed that many proteins, such as those in focal adhesions that attach the cell to the substrate, might look colocalized at the diffraction limit are definitely not colocalized at high resolution [42] (Figure 17B). With Bob Tijan's group at Janelia, we were able to show a mechanism of gene silencing, where core promoters (green) are spatially segregated from genes that hug up against the nuclear membrane [43] (red, Figure 17C). With Tom Blanpied's group at Maryland we were able to look at live cultured neurons by sptPALM (single particle tracking PALM [44]) and show that the actin that gives rise to the shapes of dendritic spines is only polymerized at certain discrete locations [45].

At the same time, Harald, being the better physicist than I am, built the ultimate PALM microscope that uses a three-phase interfereometer he originally developed in industry to measure the fly height of recording heads above a magnetic disk, that has even better sensitivity in z than in x and y [46]. He and Gleb then worked with Clare Waterman's group at NIH to unravel the entire architecture of focal adhesion proteins vertically from the substrate up to the actin cytoskeleton [47]. In a recent paper with Jennifer's group, they were able to resolve a question about ESCRT proteins which are involved in HIV

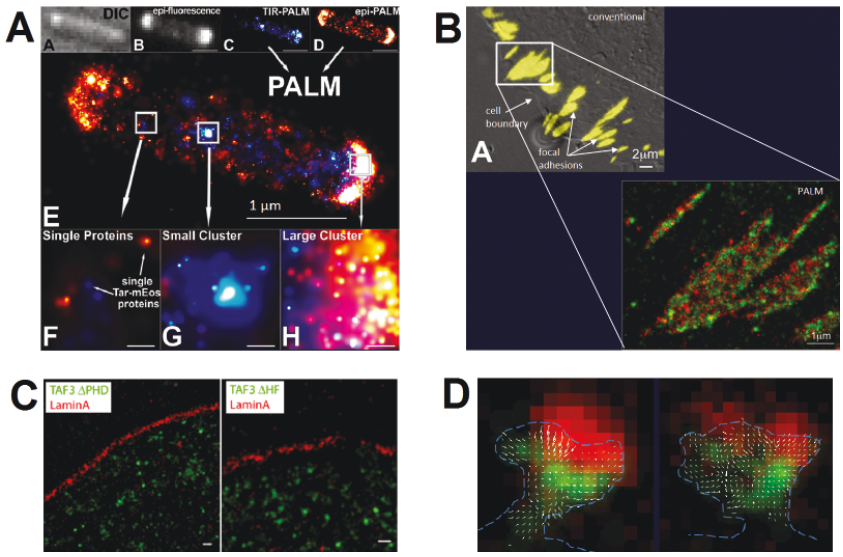

FIGURE 17. Applications of PALM. (**A**) Chemotaxis receptor clusters in *E. coli* [41]. (**B**) Two-color diffraction-limited and PALM views of the spatial relationship of vinculin and paxillin in focal adhesions [42]. (**C**) Spatial relationship of core promoters of transcription (green) relative to the nuclear membrane (red) at different stages of myogenesis [43]. (**D**) Tracks of actin polymerization in dendritic spines of live cultured neurons [45].

budding—whether these act outside of the bud or inside of the bud—and they showed that the latter is true [48].

Harald has a lot of background from his time in industry in electron microscopy, so he's also worked at correlating electron microscopy with PALM in three dimensions—in one case, looking at mitochondrial nucleoids and their location inside of the mitochondria [49], and in another looking at clathrin-coated pits [50].

I think a lot of my success is attributable to the fact that I'm a pessimist. I like to focus on problems because I think problems are opportunities. Therefore, I'd like to say a bit about what are the problems with super-resolution microscopy instead of extolling its virtues. First is that, as I said earlier, based on the Nyquist criterion you need an insanely high density of labels [51] (Figure 12). These can cause overexpression of proteins to get to those levels (Figure 18A), or if you use exogenous dyes it's hard enough to get enough specificity without a bunch of background (Figure 18B). Second, ninety-five percent of what we look at in super-resolution is fixed cells, but it's known that chemical fixatives alter the

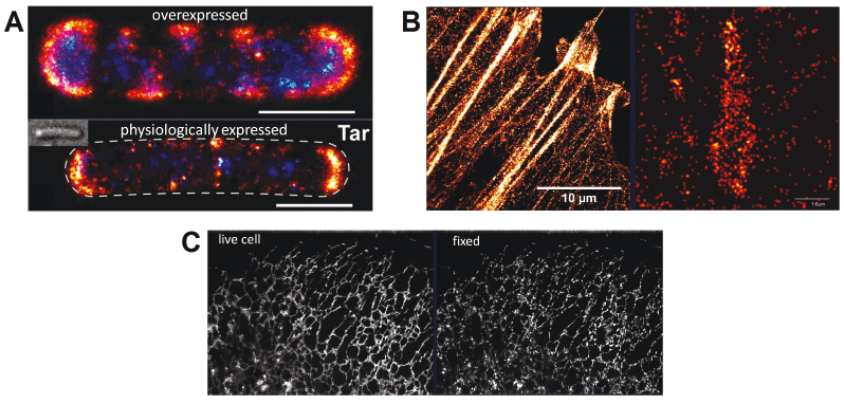

FIGURE 18. Problems in super-resolution. (**A**) Overexpression of the target protein can alter the physiological state of the cell [41]. (**B**) Exogenously-introduced dyes often have limited affinity for the desired target (left, actin cytoskeleton) and high residual background (right, focal adhesion). (**C**) Chemical fixation can alter the ultra-structure, as seen here in the endoplasmic reticulum, before and after fixation.

ultrastruture at the nanoscale (Figure 18C), so we have to put an asterisk next to almost everything that we learn by chemical fixation. These problems must be confronted by all super-resolution methods, not just PALM.

In what I think was a very important innovation a year ago was to get around the labeling density problem, Jan Ellenberg's group studied the nuclear pore. Even though it was difficult to get perfect labeling of every structure, by looking at thousands of these stereotypical structures, they could do particle averaging techniques borrowed from cryo-electron microscopy, and then were able to determine the radial positions of several key proteins in the nuclear pore to less than 1 nm by super-resolution optics [52]. There was an ambiguity in the cryo-EM data as to which way a subunit was oriented inside the pore, and that was addressed by super-resolution microscopy in this way. A really great example.

Of course, we heard in Sven Lidin's introduction that that the real promise of super-resolution, though, is the ability and the hope to look at living cells. But it's still largely a promise. Even though there have been technical demonstrations, there's been very little in terms of, I'd say, real biology learned. One problem is that if you want to get to higher and higher resolution, you have to collect many more photons than you've ever had to do at the diffraction limit (Figure 19, second column). Another is that life evolved under a solar flux of one-tenth of a watt per square centimeter. The super-resolution methods we're talking about

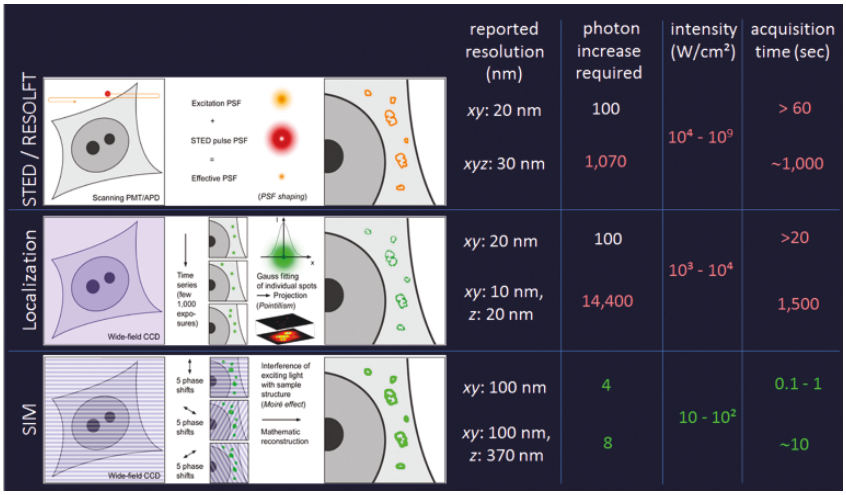

FIGURE 19. Problems in live cell super-resolution. Compared to diffraction-limited live imaging techniques, the various super-resolution methods [53] require: large increases in the amount of the fluorescence the specimen must produce (leading to rapid photobleaching); much higher illumination intensities (leading to rapid phototoxicity); and much longer acquisition times (leading to motion-induced artifacts and restricting investigations to slow dynamic processes).

today require intensities anywhere from a gigawatt per square centimeter to a kilowatt per square centimeter (third column). You have to ask yourself what are you doing to the poor cell when you're trying to look at it live? Finally, the acquisition times of many of these methods (fourth column) are far slower than the rate at which dynamics is happening in cells, so you get motion-induced artifacts or can't follow the thing you want to do.

The one technique which can do much better, because it doesn't offer as much resolution gain, is SIM [54] (structured illumination microscopy). It usually gets only twice beyond the diffraction limit, but it really offers a lot of other benefits, particularly for live imaging [55] (Figure 20). It's a shame that you can't have four people win a Nobel Prize, because I think SIM is totally justified to be a part of this.

One of the pioneers of this technology was Swedish native Mats Gustafsson, who eventually became my colleague at Janelia, before passing away from a brain tumor in 2011. We've been working with Mats' SIM technique for a while now, and eventually found ways to push beyond this 100 nm barrier, first to 80 nm and, with nonlinear SIM [57], down to 60 nm, while still capable of looking

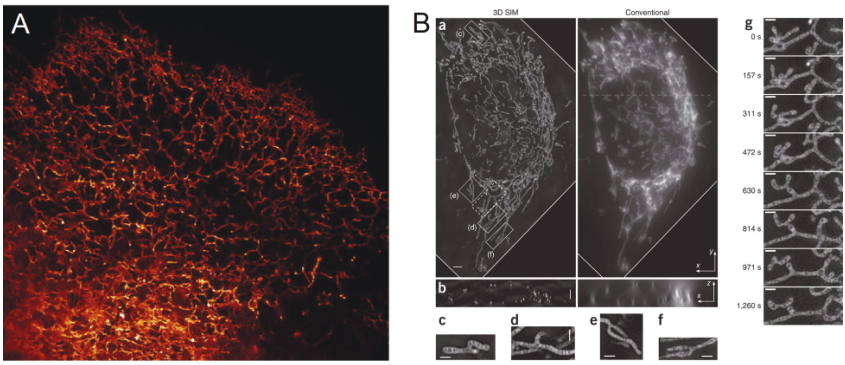

FIGURE 20. Structured illumination microscopy excels for live imaging. (**A**) 2D live SIM image of the endoplasmic reticulum in an LLC-PK1 cell, taken from a movie of 1800 time points at 0.75 sec intervals [56]. (**B**) 3D live SIM images of mitochondria in a HeLa cell, showing internal structure and the dynamics of fission/fusion events [55].

at the dynamics of living cells. I think PALM is a great tool to image structure at the nanoscale, but I think SIM is going to be the real winner for being able to look at the dynamics of living cells beyond the diffraction limit.

Despite this, it's still true that no matter what you do, and no matter what method you want to use, the higher the spatial resolution you want to have, the more measurements you have to take, which takes more time, and means throwing more potentially damaging light at the cell. The moral of the story of SIM is that by backing off a bit in terms of the resolution we're asking for, we can learn a lot more about cellular dynamics.

So, what if we back off all the way to the diffraction limit? Why would you want to do that? Well, the hallmark of life is that it's animate. Every living thing is a complex thermodynamic pocket of reduced entropy through which matter and energy is flowing continuously. While structural imaging will always be important, a complete understanding of life requires high resolution imaging across all four dimensions of space-time at the same time. So another focus of my group has been to push in this direction of 4D imaging. Over the last 10 years, there's been tremendous growth in light sheet microscopy [58]. We've adapted to this concept the idea of using non-diffracting beams, and particularly optical lattices, which was the crazy idea I was working on before Harald and I dropped it for PALM. Adapted instead to light sheet microscopy, now we have a wonderful tool to look at high-speed 3D dynamics of everything from single molecules to whole embryos over four orders of magnitude of space and time by this method, noninvasively, for very long periods of time [59] (Figure 21).

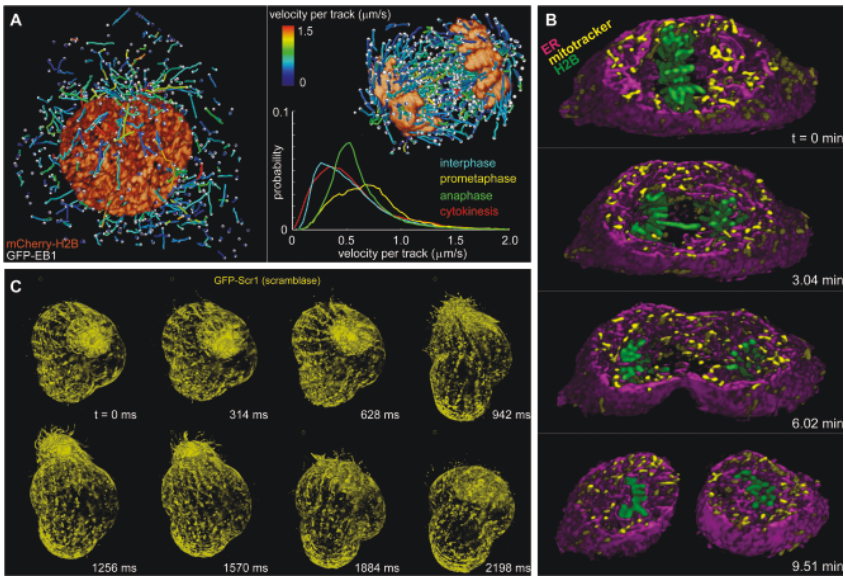

FIGURE 21. Rapid, noninvasive 3D live imaging with lattice light sheet microscopy [59]. (**A**) Tracks denoting the plus ends of growing microtubules, color coded by velocity, during different stages of mitosis, seen in relationship to chromosomes (orange). (**B**) Computationally extracted slices at different time points through a dividing LLC-PK1 cell, showing the 3D spatial relationship of chromosomes (green), endoplasmic reticulum (magneta), and mitochondria (yellow). (**C**) Rapid 3D shape changes in the protozoan *Tetrahymena thermophila* at 0.31 sec intervals.

That got us back, finally, to super-resolution, because in the same year that we published the PALM paper, Robin Hochstrasser's group published a different way of doing single-molecule localization, which doesn't involve photo-activation, but just the transient binding of molecules to cells [60]. The advantage of this method is you can have your whole imaging bath labeled with fluorophores that just keep coming, so you have an infinite army of molecules and can get higher and higher localization density. By pushing in that direction with our lattice light sheet, which allows us to get high signal-to-noise, single molecule imaging, even in the background of all of these molecules in the bath, we've been able to take 3-D localization microscopy up about two orders of magnitude in the number of localizations you can get. Plus we can look at much thicker samples than with wide-field localization, such as a whole dividing cell about 15 microns thick, where we localized 300 million distinct molecules.

The final challenge going forward is how to take cell biology away from the cover slip. That's not where cells evolved, we need to look at them inside the whole organism. The problem is that the light rays are scrambled as you go in, and so we're now borrowing adaptive optics techniques first developed by astronomers to make ground-based telescopes have resolution as good as or better than the Hubble space telescope. Moving deep into the brain of a living zebrafish embryo with this adaptive optics technique, we see low resolution and weak signal with the adaptive optics off (Figure 22A). That's what you would see with a normal microscope. Then, when we turn the adaptive optics on (Figure 22B,C), we see the recovered performance when we correct for the aberrations and return to diffraction-limited resolution [61]. Such recovery is possible even

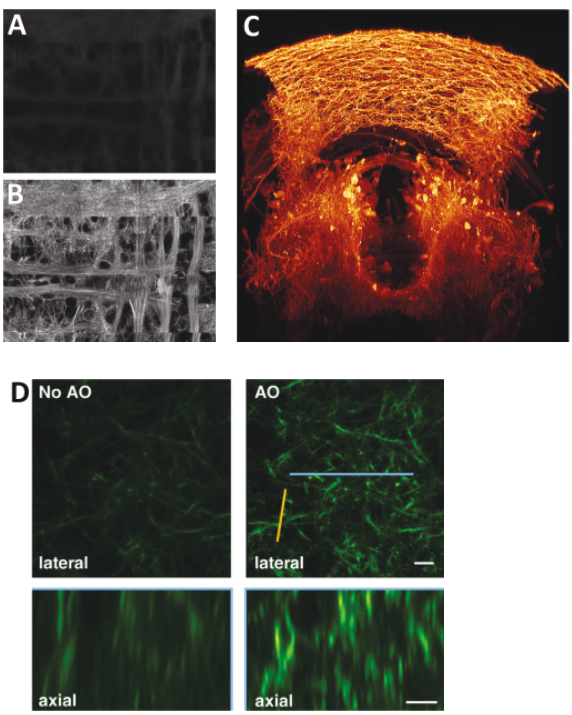

FIGURE 22. Adaptive optics enables deep imaging at high resolution. (**A**) Two-photon image of membrane-labeled neurons in the spinal cord of a live zebrafish embryo, 72 hours post fertilization. (**B**) Same region after adaptive optical correction using direct wavefront sensing [61], demonstrating recovery of signal and spatial resolution. (**C**) Adaptive optical correction of a sparse subset of neurons over a large portion of the zebrafish brain. (**D**) In vivo two-photon lateral and axial views of neural processes deep in the mouse cortex, before (left) and after (right) adaptive optical correction using indirect wavefront sensing [63].

in the scattering brain tissue of the mouse (Figure 22D) [62–64]. The ultimate goal of my group is to try to combine these technologies to be able to look deep in a multi-cellular context, to be able to look noninvasively and fast with methods like lattice light sheet, and then bring in super-resolution techniques such as SIM and PALM to then add the high spatial resolution on top of that. At that point, I'm done and I'm out of microscopy and I'll be back into that black phase and trying to figure out something else that I want to do.

I'd just like to end with a couple of things. First, there are many, many people to thank, but the guy I have to single out is Harald (Hess). I would have flamed out of Bell Labs in my first few years if I hadn't latched onto him as a friend and a mentor. There's no way I would've had the courage to pursue PALM on my own without him by my side. One of the bittersweet things about winning this award is not having him here by my side up on the stage. But I feel this award is very much as much his as it is mine.

The last thing I would like to say is a lot of what you heard this morning, like in Shuji Nakamura's talk and Stefan Hell's talk, and my talk, is about taking risks. People are always exhorted to take risks, and that's fine. But you're hearing that from guys whose risks paid off. It's not a risk unless you fail most of the time. And so I'd like to dedicate my talk to all of the unknown people out there in any walk of life who have gambled their fortunes, their careers, and their reputations to take a risk but, in the end, failed. I'd just like to say that they should remember that it's the struggle itself that is its own reward, and the satisfaction that you knew that you gave everything you had to make the world a better place. Thank you for your time.

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