Nobel Lecture: Nanoscopy with freely propagating light*

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We are all familiar with the sayings "a picture is worth a thousand words" and "seeing is believing." Not only do they apply to our daily lives, but certainly also to the natural sciences. Therefore, it is probably not by chance that the historical beginning of modern natural sciences very much coincides with the invention of light microscopy. With the light microscope mankind was able to see for the first time that every living being consists of cells as basic units of structure and function; bacteria were discovered with the light microscope and also mitochondria as examples of subcellular organelles.

However, we learned in high school that the resolution of a light microscope is limited to about half the wavelength of the light in use (Verdet, 1869; Abbe, 1873; von Helmholtz, 1874; Rayleigh, 1896) which typically amounts to about 200–350 nm (Fig. 1). If we want to see details of smaller things, such as viruses, for example, we have to resort to electron microscopy. Electron microscopy has achieved a much higher spatial resolution—tenfold, hundredfold, or even thousandfold higher, in fact, down to the size of a single molecule. Therefore the question comes up: Why do we care for the light microscope and its spatial resolution, now that we have the electron microscope?

The answer to this question is given in Fig. 2, where I've conducted a small "experiment." I counted the numbers of papers published in this issue of *Nature Medicine* where a light microscope was used, and where an electron microscope was used. The clear winner was light microscopy, which has remained the most popular microscopy technique in the life sciences. This is for two strong reasons.

The first reason is that light microscopy is the only way in which we can look inside a living cell, or even living tissues, in three dimensions; it is minimally invasive. But, there is another reason. When we look into a cell, we are usually interested in a certain species of proteins or other biomolecules, and we have to make this species distinct from the rest—we have to "highlight" those proteins (Alberts *et al.*, 2002). This is because, to light or to electrons, all the proteins look the same.

In light microscopy this "highlighting" is readily feasible by attaching a fluorescent molecule to the biomolecule of interest (Giepmans *et al.*, 2006). Importantly, a fluorescent molecule (Fig. 2) (Lakowicz, 2006) has, among others, two fundamental states: a ground state and an excited fluorescent state with higher energy. If we shine light of a suitable wavelength on it, for example, green light, it can absorb a green photon so that the molecule is raised from its ground state to the excited state. Right afterward the atoms of the molecule wiggle a bit—that is why the molecules have vibrational substates—but within a few nanoseconds, the molecule relaxes back to the ground state by emitting a fluorescence photon.

Because some of the energy of the absorbed (green) photon is lost in the wiggling of the atoms, the fluorescence photon is redshifted in wavelength, shown as orange in Fig. 2. This is actually very convenient, because we can now easily separate the fluorescence from the excitation light, the light with which the cell is illuminated. This shift in wavelength makes fluorescence microscopy extremely sensitive. In fact, it can be so sensitive that one can detect a single molecule, as has been discovered through the works of my co-laureate W. E. Moerner (Moerner and Kador, 1989), of Michel Orrit (Orrit and Bernard, 1990), and their co-workers.

However, if a second molecule, a third molecule, a fourth molecule, a fifth molecule, and so on are positioned closer together than about 200–350 nm, we cannot tell them apart, because they appear in the microscope as a single blur. Therefore, it is important to keep in mind that resolution is about telling features apart; it is about distinguishing them.

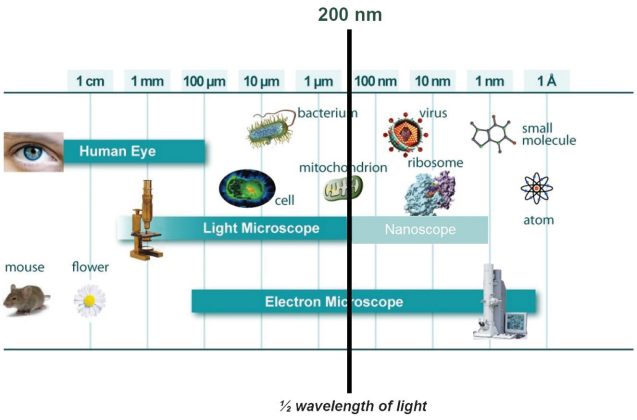

FIG. 1 (color). Length scales and spatial resolution limits of visual inspection (human eye), light (optical) microscopy, and electron microscopy. Far-field optical nanoscopy extends the resolution much beyond Abbe's limit of half the wavelength of light (> 200 nm), down to the single-digit nanometer range.

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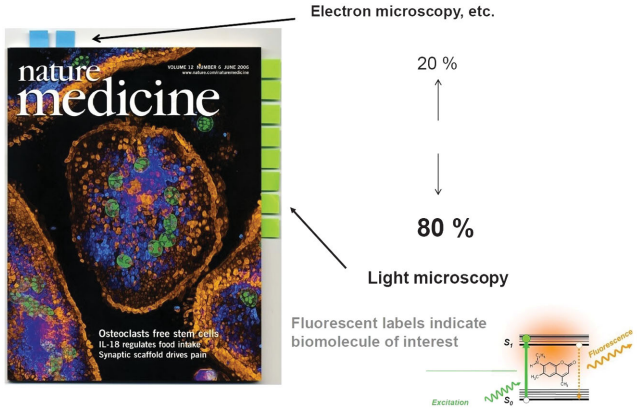

FIG. 2 (color). Light microscopy remains the most popular microscopy method in the life sciences, due to a number of distinct advantages such as live-cell imaging and biomolecular specificity. The latter is provided by labeling the biomolecules of interest with fluorescent markers, allowing their species-specific detection in the microscope.

Resolution must not be confused with sensitivity of detection, because it is about seeing different features as separate entities.

Now it is easy to appreciate that a lot of information is lost if we look into a cell with a fluorescence microscope: anything that is below the scale of 200 nm appears blurred. Consequently, if one manages to come up with a focusing (far-field) fluorescence microscope which has a much higher spatial resolution, this would have a tremendous impact in the life sciences and beyond.

In a first step, we have to understand why the resolution of a conventional light-focusing microscope is limited. In simple terms it can be explained as follows. The most important element of a light microscope is the objective lens (Fig. 3). The role of this objective lens is simply to concentrate the light in space, to focus the light down to a point. However, because light propagates as a wave, it is not possible for the lens to concentrate the light in a single point. Rather the light will be diffracted, "smeared out" in the focal region, forming a spot of light which is—at minimum—about 200 nm wide and about 500 nm along the optical axis (Born and Wolf, 2002). This has a major consequence: if several features fall within this region, they will all be flooded with this light at the same time and hence produce signal simultaneously. In the case of fluorescence microscopy, this is the excitation light. As we try to detect the fluorescence signal with a lens and relay it onto a detector, the signals produced by the molecules within this >200 nm spot will be confused. This is because at the detector, each molecule will also produce a spot of focused (fluorescence) light and the spots from these simultaneously illuminated molecules will overlap (Fig. 3). No detector will be able to tell the signal from these molecules apart, no matter if it is the eye, a photomultiplier, or even a pixelated camera.

The person who fully appreciated that diffraction poses a serious limit on the resolution was Ernst Abbe (Fig. 4), who lived at the end of the 19th century and who coined this "diffraction barrier" in an equation which has been named after him (Abbe, 1873). It says that, in order to be separable, two features of the same kind have to be farther apart than the wavelength divided by twice the numerical aperture of the objective lens. One can find this equation in most textbooks of physics or optics, and also in textbooks of biochemistry and molecular biology, due to the enormous relevance of light microscopy in these fields. Abbe's equation is also found on a memorial which was erected in Jena, Germany, where Ernst Abbe lived and worked, and there it is written in stone. This is what scientists believed throughout the 20th century. However, not only did they believe it, it also was a fact. For example, if one wanted to look at features of the cellular cytoskeleton in the 20th century (Alberts *et al.*, 2002) this was the type of resolution obtained (Fig. 4, "Confocal"). But now, today, we get the resolution shown in Fig. 4 ("STED") and this resolution has become a new standard. So what I describe in this lecture is how this transition was made, from the previous

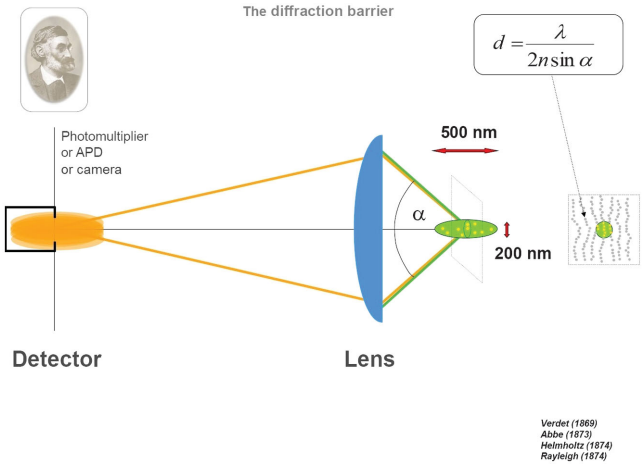

FIG. 3 (color). Focusing of light by the microscope (objective) lens cannot occur more tightly than the diffraction (Abbe's) limit. As a result, all molecules within this diffraction-limited region are illuminated together, emit virtually together, and cannot be told apart.

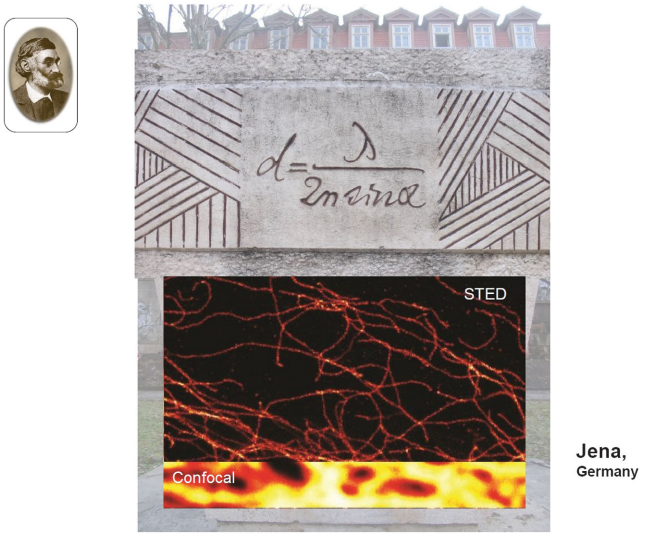

FIG. 4 (color). The diffraction resolution limit carved in stone (top: memorial in honor of Ernst Abbe in Jena, Germany), and resolution increase brought about by STED nanoscopy (top of recording) over confocal imaging (bottom) when imaging the cytoskeleton in a cell.

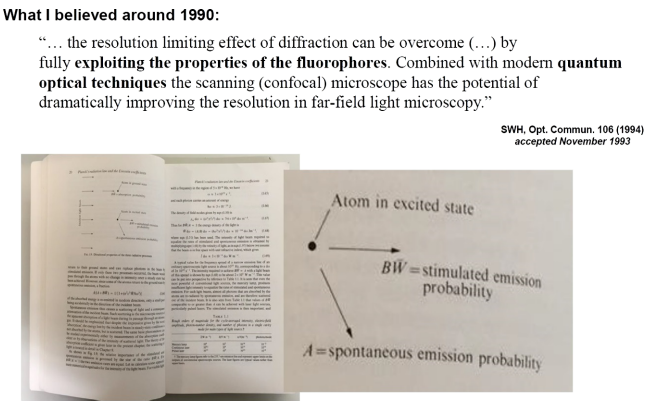

FIG. 5 (color online). Realization in the early 1990s that the key to surpassing the diffraction resolution limit lies in fluorophore properties (quote from a manuscript submitted in 1993, top). The photograph (bottom) shows page 20 of the book "The Quantum Theory of Light" by Rodney Loudon (Oxford Science Publications), where I found a reminder of the phenomenon of stimulated emission, which I, of course, knew about from my physics studies, on Saturday morning, 6 November 1993. My copy of the book is now on display at the Nobel Museum, Stockholm.

diffraction-limited resolution to resolution far beyond the diffraction barrier.

It started out in the late 1980s. I was a student in Heidelberg in those days, and I worked in the research area of light microscopy, so I was of course familiar with Abbe's equation. I began wondering: This equation was coined in 1873, and yet it is now 1990. So much new physics emerged during the 20th century and so many new phenomena were discovered—as a matter of fact, I had to learn so much for my examinations! There should be phenomena—at least one—that could be utilized to overcome the diffraction barrier in a light microscope operating with propagating beams of light and regular lenses. Well, I understood that it won't work just by changing the way the light is propagating, the way the light is focused. [Actually I had looked into that; it led me to the invention of the 4Pi microscope (Hell and Stelzer, 1992; Hell, Schrader, and Van der Voort, 1997) which improved the axial resolution, but did not overcome Abbe's barrier.] I was convinced that a potential solution must have something to do with the major discoveries of the 20th century: quantum mechanics, molecules, molecular states, and so on.

Therefore, I started to check my textbooks again in order to find something that could be used to overcome the diffraction barrier in a light-focusing microscope. One day I put my ideas about solving the problem down in writing (Fig. 5). In simple terms, the idea was to check out the spectroscopic properties of fluorophores, their state transitions, and so on, specifically to solve the resolution problem. Until then, they had been used only for fluorescence signal generation or to measure pH or calcium concentrations, etc. But, maybe there was a property that could be used for the purpose of making Abbe's barrier obsolete. Alternatively, there could be a quantum-optical effect whose potential has not been realized, simply because nobody thought about overcoming the diffraction barrier (Hell, 1994).

With these ideas in mind, one day when I was not very far from here in Åbo/Turku, just across the Gulf of Bothnia, on a Saturday morning, I browsed a textbook on quantum optics (Loudon, 1983) and stumbled across the page shown in Fig. 5. It dealt with stimulated emission. All of a sudden I was electrified. Why?

To reiterate, the problem is that the lens focuses the light in space, but not more tightly than 200 nm. All the features within the 200 nm region are simultaneously flooded with excitation light. This cannot be changed, at least not when using conventional optical lenses. But perhaps we can change the fact that all the features which are flooded with (excitation) light are, in the end, capable of sending light (back) to the detector. If we manage to keep some of the molecules dark—to be precise, put them in a nonsignaling state in which they are not able to send light to the detector—we will see only the molecules that can, i.e., those in the bright state. Hence, by registering bright-state molecules as opposed to dark-state molecules, we can tell molecules apart. So the idea was to keep a fraction of the molecules residing in the same diffraction area in a dark state, for the period of time in which the molecules residing in this area are detected. In any case, keep in mind: the state (transition) is the key to making features distinct. And resolution is about discerning features.

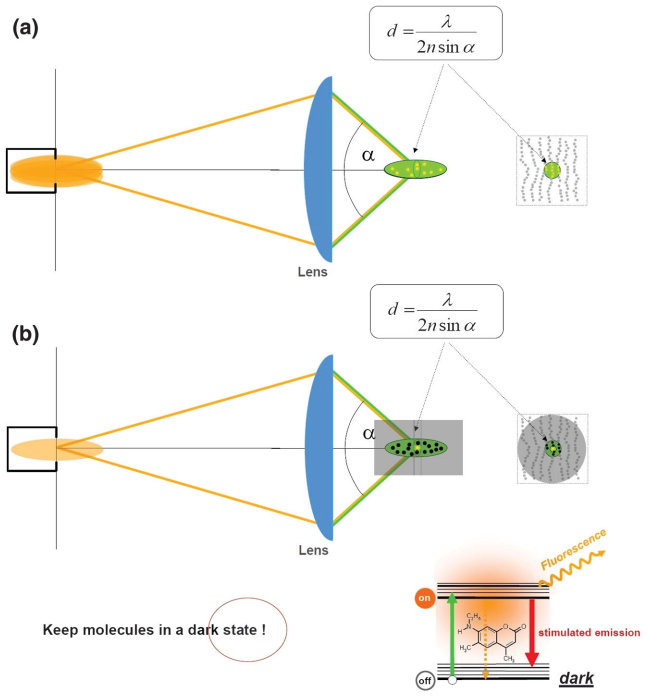

FIG. 6 (color). Switching molecules within the diffraction-limited region transiently "off" (i.e. effectively keeping them in a nonsignaling state) enables the separate detection of neighboring molecules residing within the same diffraction region. (a) In fluorescence microscopy operating with conventional lenses (e.g. confocal microscopy), all molecules within the region covered by the main diffraction maximum of the excitation light are flooded with excitation light simultaneously and emit fluorescence together. This is because they are simultaneously allowed to assume the fluorescent (signaling) state. (b) Keeping most molecules–except the one(s) one aims to register–in a dark state solves the problem. The dark state is a state from which no signal is produced at the detector. Such a transition to the dark "off" state is most simply realized by inducing stimulated emission, which instantaneously forces molecules to their dark ("off") ground state.

For this reason, the question comes up: are there dark states in a fluorescent molecule? The answer has actually been given in the energy diagram shown in Fig. 2, reiterated in Fig. 6(b). The ground state of the fluorophore is a dark state! For the molecule to emit fluorescence, the molecule has to be in its excited state. So the excited state is the signaling bright state, but the ground state is, of course, a nonsignaling dark state.

What is now the role of stimulated emission? Actually, the answer is as simple as profound: it makes dark molecules, that is, molecules that are not seen by the detector! This was the reason why I was so excited. I had found a way to make normal fluorophores not fluoresce, just normal fluorophores that were commonly used in fluorescence microscopy. And now you can easily envisage how the microscope works: stimulated emission depletion—or STED—microscopy (Hell and Wichmann, 1994; Klar and Hell, 1999; Klar *et al.*, 2000; Dyba and Hell, 2002; Westphal and Hell, 2005; Donnert *et al.*, 2006; Willig *et al.*, 2006; Westphal *et al.*, 2008; Berning *et al.*, 2012). Figure 7(a) sketches the lens, the critical component of a far-field optical microscope, as well as a sample and a detector. We use a beam of light for exciting molecules from the ground state to the excited state, to make them bright ("ON"), i.e., get them to the excited state. Inevitably, the excitation light will be diffracted and one obtains a spot of light of at least 200 nm. Signal which is produced therein, from all the molecules, will be able to end up at the detector. But now, we use a second beam of light which induces stimulated emission and thus makes dark-state molecules. The idea is to instantly "push" the molecules that were excited back down to the ground state so that the molecule is not capable of emitting light, because it has assumed the dark ground state ("OFF").

The physical condition for achieving this is that the wavelength of the stimulating beam is longer [Fig.7(c)]. The photons of the stimulating beam have a lower energy, so as not to excite molecules but to stimulate the molecules going from the excited state back down to the ground state. There is another condition, however: we have to *ensure* that there is indeed a red photon at the molecule which pushes the molecule down. I am saying this because most photons pass by the molecules, as there is a finite interaction probability of the photon with a molecule, i.e., a finite cross section of interaction. But if one applies a stimulating light intensity at or above a certain threshold, one can be sure that there is at least one photon which "kicks" the molecule down to the ground state, thus making it instantly assume the dark state.

Figure 7(d) shows the probability of the molecule to assume the bright state, the S_1, in the presence of the STED beam transferring the molecule to the dark ground state. Beyond a certain threshold intensity, I_s, the molecule is clearly turned "off." One can apply basically any intensity of green light. Yet, the molecule will not be able to occupy the bright state and thus not signal. Now the approach is clear: we simply modify this red beam to have a ring shape in the focal plane (Willig *et al.*, 2006; Keller, Schoenle, and Hell, 2007) such that it does not carry any intensity at the center. Thus, we can turn off the fluorescence ability of the molecules everywhere but at the center. The ring or "doughnut" becomes weaker and weaker toward the center, where it is ideally of zero intensity. There, at the center, we will not be able to turn the molecules off, because there is no STED light, or it is much too weak.

Now let's have a look at the sample [Fig. 7(b)] and let us assume that we want to see just the fiber in the middle. Therefore, we have to turn off the fiber to its left and the one to its right. What do we do? We cannot make the ring smaller, as it is also limited by diffraction. Abbe would say "Making narrower rings of light is not possible due to diffraction." But

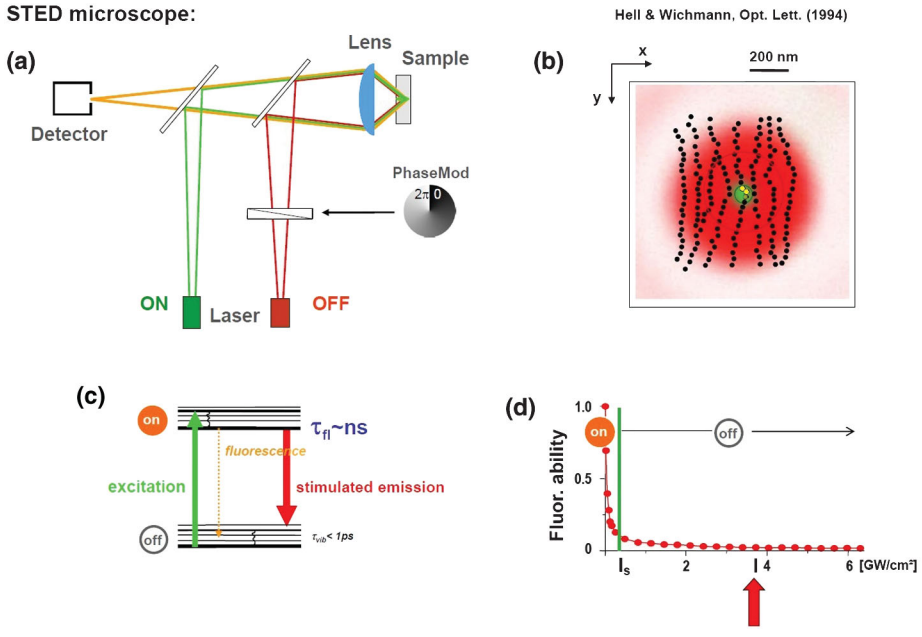

FIG. 7 (color). STED microscopy. (a) Setup schematic. (b) Region where the molecule can occupy the "on" state (green) and where it has to occupy the "off" state (red). (c) Molecular transitions. (d) For intensities of the STED light (red) equaling or in excess of the threshold intensity I_s, molecules are effectively switched "off." This is because the STED light will always provide a photon that will stimulate the molecule to instantly assume the ground state, even in the presence of excitation light (green). Thus, the presence of STED light with intensity greater than I_s switches the ability of the molecules to fluoresce off.

we do *not* have to do that. Rather, we simply have to "shut off" the molecules of the fibers that we do *not* want to see, that is, we make their molecules dwell in a dark state, until we have recorded the signal from that area. Obviously, the key lies in the preparation of the states. So what do we do? We make the beam strong enough so that the molecules even very close to the center of the ring are turned "off" because they are effectively confined to the ground state all the time. This is because, even close to the center of the ring, the intensity is beyond the threshold I_s in absolute terms.

Now we succeed in separation: only in the position of the doughnut center are the molecules allowed to emit, and we can therefore separate this signal from the signal of the neighboring fibers. And now we can acquire images with subdiffraction resolution: we can move the beams across the specimen and separate each fiber from the other, because their molecules are forced to emit at different points in time. We play an *on/off game*. Within the much wider excitation region, only a subset of molecules that are at the center of the doughnut ring are allowed to emit at any given point in time. All the others around them are effectively kept in the dark ground state. Whenever one makes a check which state they are in, one will nearly always find those molecules in the ground state.

This concept turned out to work very well (Klar *et al.*, 2000; Westphal and Hell, 2005; Willig *et al.*, 2006; Göttfert *et al.*, 2013). Figure 8(a) contains a standard, high-end confocal recording of something which one cannot make out what it is. Figure 8(b) shows the same region imaged using STED microscopy. The resolution is increased by about an order of magnitude (in the red channel), and one can clearly discern what is actually being imaged here: nuclear pore complexes. As a result of the high resolution, you can see that this nuclear pore complex features eight molecular subunits. The eightfold symmetry comes out very clearly (Göttfert *et al.*, 2013). There is almost no comparison with the standard confocal recording.

Needless to say, if afforded this increase in spatial resolution, one obtains new information. In other words, new insights are gained with this microscope. I briefly describe research done in collaboration with virologists interested in the human immunodeficiency virus (HIV). Generally, viruses are about 30 to 150 nm in diameter (Alberts *et al.*, 2002). So, if one wants to image them with a light microscope … there is no chance this will succeed—one will not see any details of protein distributions on the virus particles. A diffraction-limited fluorescence microscope would yield just a 250–350 nm sized fluorescence blur. The HIV is about 140 nm in size. The scientists collaborating with us were interested in finding out how a protein called Env is distributed on the HIV particle (Chojnacki *et al.*, 2012), Fig. 9. In the normal recording, nothing specific is seen. In contrast, the high-resolution STED recording revealed that the protein Env forms patterns on the HIV particles. What has actually been found out in this study is that the mature HIV particles—those which are ready to infect the next cell—have the Env concentrated basically in a single place on the virus. It seems to be a requirement for HIV to be very effective. This is an example how new mechanistic insight was gained as a result of subdiffraction-resolution imaging.

Of course, a strength of light microscopy is that we can image living cells. Figure 10 shows a video-rate recording

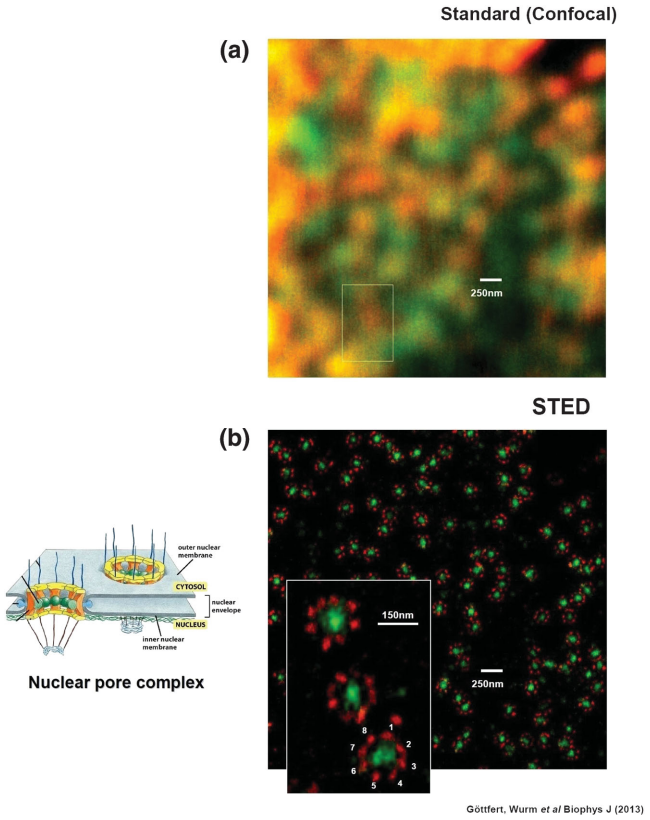

FIG. 8 (color). Nuclear pore complex architecture in an intact cell nucleus imaged by (a) confocal microscopy (diffraction-limited), and (b) STED nanoscopy.

with STED microscopy. These are synaptic vesicles in the axon of a living neuron (Westphal *et al.*, 2008). One can directly see how they move about and we can study their dynamics and their fate over time. It is clearly important to be able to image living cells.

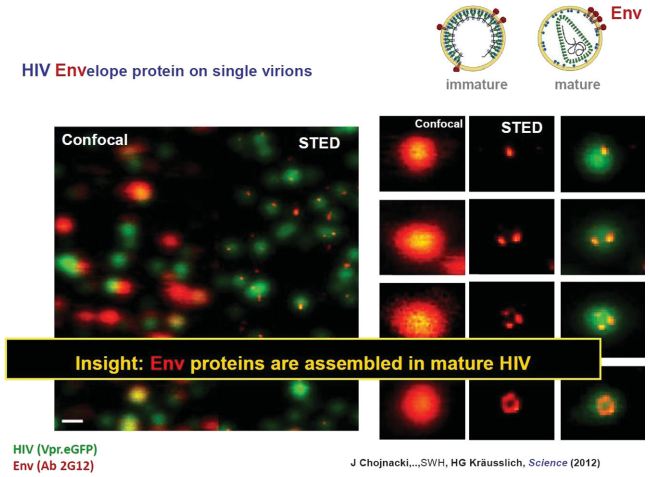

FIG. 9 (color). STED nanoscopy of the HIV Envelope protein Env on single virions. Confocal microscopy is not able to reveal the nanoscale spatial distribution of the Env proteins; the images of the Env proteins on the virus particles look like 250–350 nm sized blurred spots (orange, left column). STED microscopy reveals that the Env proteins form spatial patterns (center column, orange), with mature particles having their Env strongly concentrated in space (panel in top row of center column, orange).

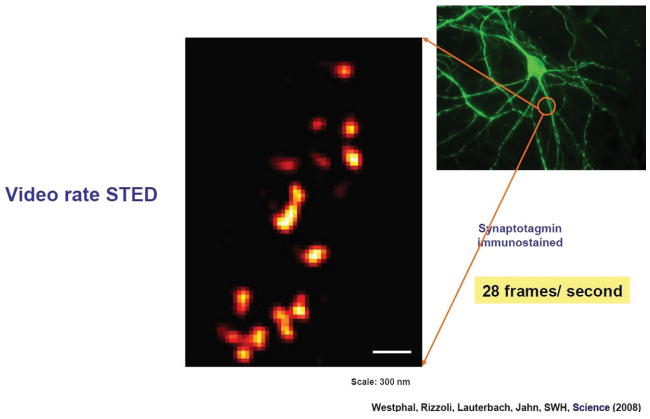

FIG. 10 (color). Video-rate STED imaging of synaptic vesicle motion in axon of living hippocampal neuron. (Lecture contains movie.)

Live-cell imaging "at the extreme" is pictured in Fig. 11. Here, we opened the skull of an anesthetized mouse and looked into the brain of the mouse at the upper, so-called molecular layer of the visual cortex (Berning *et al.*, 2012). This was a transgenic mouse, meaning that some of its neurons expressed a fluorescent protein, specifically the yellow fluorescent protein (YFP), and this is why this neuron is highlighted from the surrounding brain. The surrounding brain tissue is dark. Next we took sequential recordings and could see the receiving synaptic ends of the neuron—the so-called dendritic spines. They move slightly, and it is worthwhile zooming in on them. One discerns the spine neck and, in particular, the details of the cup-shaped end of the dendritic spines. STED microscopy allows these tiny morphologies to be visualized, such that we can observe their

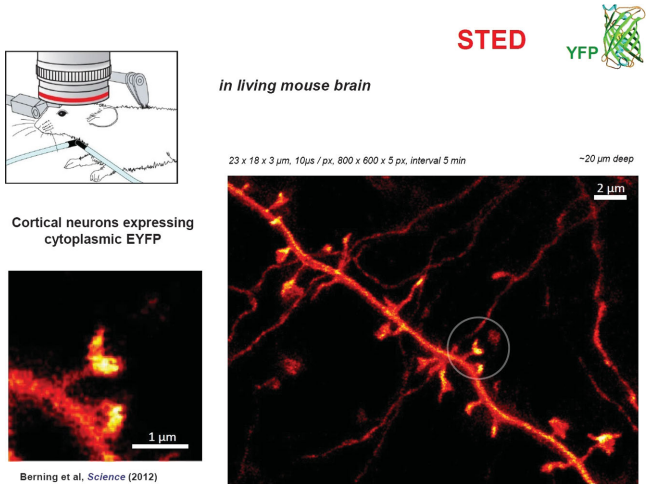

FIG. 11 (color). STED nanoscopy in living mouse brain. The recording shows a part of a dendrite of a neuron expressing a yellow fluorescent protein (EYFP) in the cytosol, thus highlighting the neuron amidst surrounding (nonlabeled) brain tissue. The 3–4 fold improved resolution over confocal and multiphoton excitation fluorescence microscopy reveals the dendritic spines (encircled) with superior clarity, particularly the cuplike shape of some of their terminals containing the receiving side of the synapses.

subtle temporal changes. I am very confident that in the not too distant future we will be able to image the proteins here at the synapse (Willig *et al.*, 2014). I can also imagine that we will be able to give a visual cue to the mouse and observe how this actually changes the protein distribution directly at the synapse. Thus, in the end we should learn how neuronal communication or memory formation works at the molecular level.

Since STED microscopy relies on freely propagating light, one can perform three-dimensional (3D) imaging. It is possible to focus into the brain tissue, for example, and record a 3D data set. Figure 12 shows a 3D superresolution recording of actin in a living neuron in a so-called organotypical hippocampal slice.

Coming back again to the basics, to the spatial resolution, some of you will ask: What is the resolution we can get? What is the limit? Indeed, is there a new limit? So let us get back to the principle. The "name of the game" is that we turn off molecules everywhere but at the intensity minimum, at the central zero, of the STED beam (Hell, 2003, 2004, 2007, 2009a). If we can make the region in which the molecules are still allowed to emit smaller, the resolution is improved; that is clear. The extent (or diameter) of the region in which the molecules are still on now determines the spatial resolution. Clearly, it cannot be described by Abbe's equation any more. In fact, this diameter must depend on the intensity I which is found at the doughnut crest [Figs. 13(b) and 13(d)] and on the threshold intensity I_s, which is a characteristic of the photon-molecule interaction. The larger their ratio becomes, the smaller d will become. It is now easy to appreciate that this ratio must be found in the denominator, if we describe the resolution with a new equation which is now obviously required (Hell, 2003, 2004; Westphal and Hell, 2005). In fact, d scales inversely with the square root of I/I_s. So the larger I/I_s, the smaller is d. As a result, d tends to 0 for larger and larger values of I/I_s [Figs. 13(b) and 13(d)].

In the situation depicted in Fig. 13(b), we cannot separate two of the close-by molecules because both are allowed to emit at the same time. But let us make the beam a bit stronger, so that only one molecule "fits in" the region in which the molecules are allowed to be on. Now the resolution limit is apparent: it is the size of a *molecule*, because a molecule is the smallest entity one can separate. This is not surprising! After all, we separate features by preparing their molecules in two

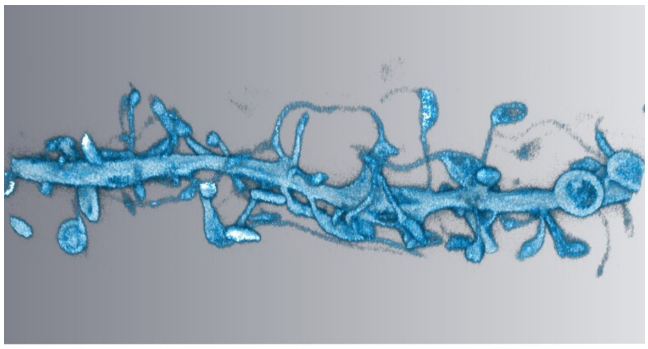

FIG. 12 (color online). Rendition of three-dimensional STED nanoscopy data showing the dendritic actin from a neuron of a living organotypical hippocampal brain slice (mouse).

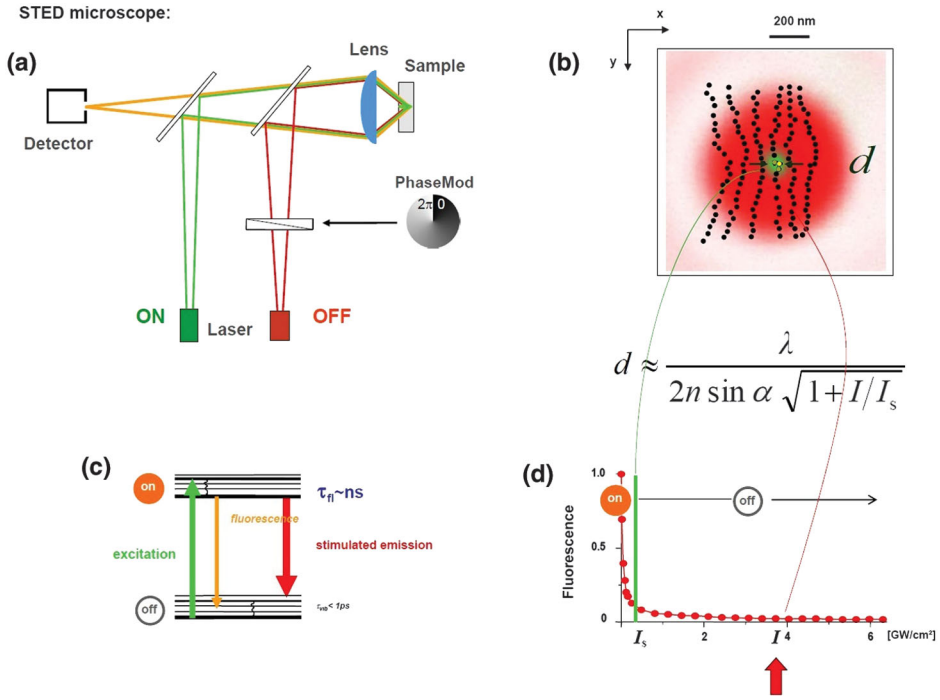

FIG. 13 (color). Resolution scaling in the STED/RESOLFT concepts: an extension of Abbe's equation. The resolution scales inversely with the square root of the ratio between the maximum intensity at the doughnut crest and the fluorophore-characteristic threshold intensity I_s.

different states, and so it must be the molecule which is the limit of spatial resolution. When two molecules come very close together, we can separate them because at the time one of them is emitting, the other one is off and vice versa (Hell, 2003, 2007, 2009a, 2009b).

It is worth noting that if all the off or dark molecules are entirely dark, i.e., nonsignaling, detecting a *single* photon from a molecule is absolutely enough to know that there is a molecule present (at the minimum of the STED beam). The position of that molecule is entirely determined by the presence of the STED-beam photons. These photons determine exactly *where* the molecule is on and where it is off (dark). The detected fluorescence photons only indicate the presence of a molecule, or many of them (Hell, 2007, 2009a, 2009b).

Does one typically obtain molecular spatial resolution, and what about in a cell? For STED microscopy right now, the standard of resolution is between 20 and 40 nm depending on the fluorophore, and depending on the fluorophore's chemical environment (Göttfert *et al.*, 2013). But this is something which is progressing; it is under continuous development. With fluorophores which have close-to-ideal properties and can be turned on and off as many times as desired, we can do much better, of course.

In fact, there are such fluorophores—not organic ones, inorganic ones—which meet this requirement already. These are so-called charged nitrogen vacancies in diamond (Fig. 14), fluorescent defects in diamond crystals which can be turned on and off an almost unlimited number of times (Rittweger *et al.*, 2009). Imaging these, we managed to squeeze down the region of emission to 2.4 nm (Wildanger *et al.*, 2012). It is worth keeping in mind that the wavelength responsible for this result is 775 nm. So the region of emission is smaller than 1%, a very small fraction of the wavelength.

This may look like a proof-of-principle experiment, and to some extent it is. But it is not just that, there is another reason why to perform these experiments (Han *et al.*, 2009; Rittweger *et al.*, 2009; Rittweger, Wildanger, and Hell, 2009). The so-called charged nitrogen vacancies are currently regarded as attractive candidates for quantum computation: as qubits operating at room temperature (Wrachtrup and Jelezko, 2006; Wrachtrup, 2010). They possess a spin state with a very long coherence time even at room temperature, which can be prepared and read out optically. Being less than a nanometer in size, they can sense magnetic fields at the nanoscale (Maze *et al.*, 2008; Wildanger, Maze, and Hell, 2011). We inherently have nanosensors in here, and STED is perhaps the best way of reading out the state and the magnetic fields at the nanoscale. In the end, this could make STED an interesting candidate perhaps for reading out qubits in a quantum computer, or who knows… . Development goes on!

Returning to the fundamentals, I emphasized that the name of the game is on/off, or keeping a fraction of the molecules dark for separation (Hell, 2007, 2009a, 2009b). This is how we separate molecules, with a bright state and a dark state. Once it is clear that this is a general principle it is obvious that stimulated emission is not the only way by which we can play this on/off game. There must also be other on and off states in a dye which one can use to the same effect (Dyba and Hell, 2002; Hell, 2003, 2004, 2007). With this in mind, I browsed other textbooks and found that there are triplet states, long-lived dark states and, of course, in chemistry textbooks, one will find that there is photo-induced cis-trans isomerization (Fig. 15). One might ask

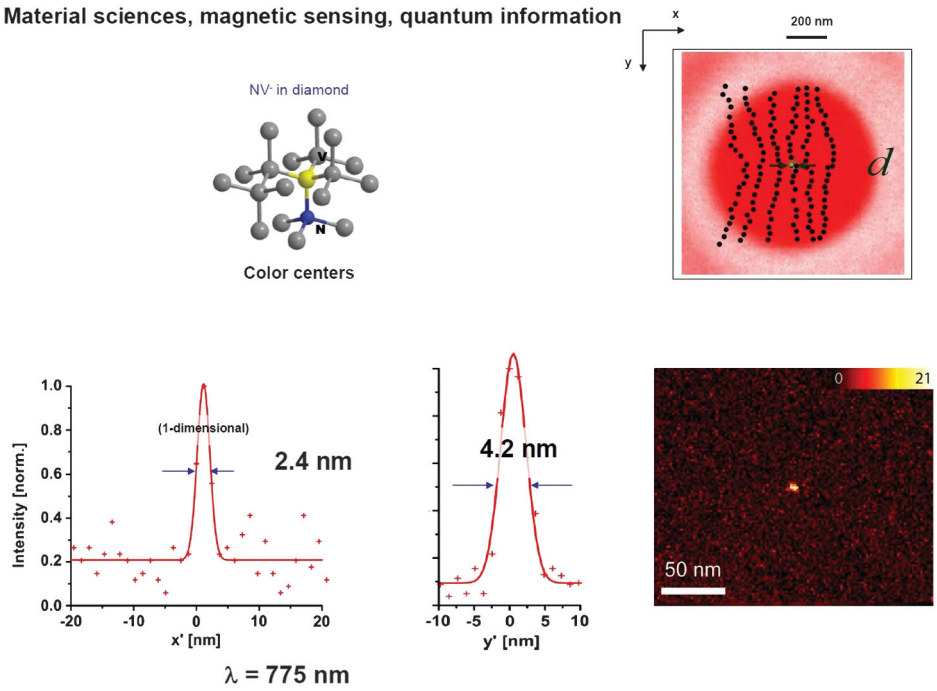

FIG. 14 (color). Fluorophores affording virtually unlimited repetitions of the resolution-enabling on-off state transitions provide the present resolution records in far-field optical imaging using STED, in the single-digit nanometer regime. Color centers (charged nitrogen vacancy centers) in diamond hold great potential for various other applications, notably in magnetic sensing and quantum information, which may be eventually read out with diffraction-unlimited spatial resolution using conventional lenses, i.e. even when packed very densely at the nanometer scale.

why use these special transitions that, unlike stimulated emission, are not found in absolutely any fluorophore, as special fluorophores are needed for this? After all, the transitions used in STED are truly basic: optical excitation and deexcitation. And the two states between which these transitions are induced are the most basic states imaginable, namely, the ground and the first excited state.

Indeed, it turns out that there is a strong reason for looking into other types of states and state transitions. Consider the state lifetimes (Fig. 15). For the basic STED transition,

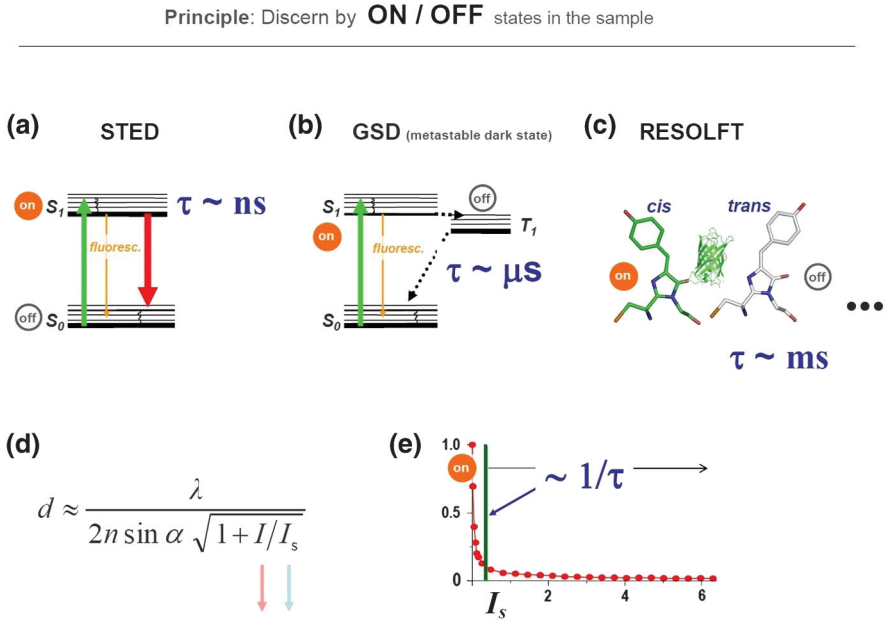

FIG. 15 (color). States and state transitions utilized in (a) STED, (b) GSD, and (c) RESOLFT nanoscopy. (d) The modified expression for the resolution describes the spatial region in which molecules can still reside in the on state. (e) The intensity I_s for guaranteeing the transition from the ON to the OFF state is inversely related to the state liftetime. The longer the lifetime of the involved states, the fewer photons per second are needed to establish the on-off state difference which is required to separate features residing within the diffraction barrier.

the lifetime of the state, the excited state, is nanoseconds [Fig. 15(a)]. For metastable dark states used in methods termed ground state depletion (GSD) microscopy (Hell and Kroug, 1995; Bretschneider, Eggeling, and Hell, 2007; Fölling *et al.*, 2008) [Fig. 15(b)] the lifetime of the state is microseconds, and for isomerization it is on the order of milliseconds [Fig. 15(c)]. Why are these major increases in the utilized state lifetime relevant?

Well, just remember that we separate adjacent features by transferring their fluorescent molecules into two different states. But if the state—one of the states—disappears after a nanosecond, then the *difference in states* created disappears after a nanosecond. Consequently, one has to hurry up putting in the photons, creating this difference in states, as well as reading it out, before it disappears. But if one has more time—microseconds, milliseconds—one can turn molecules off, read the remaining ones out, turn on, turn off ….; they stay there, because their states are long lived. One does not have to hurry up putting in the light, and this makes this "separation by states" operational at *much* lower light levels (Hell and Kroug, 1995; Hell, 2003).

To be more formal, the aforementioned intensity threshold I_s scales inversely with the lifetime of the states involved [Fig. 15(e)]: the longer the lifetime, the smaller is the I_s, and the diffraction barrier can be broken using this type of transition at much lower light levels. I_s goes down from megawatts (STED), kilowatts (GSD), down to watts per square centimeter for millisecond switching times—a 6 orders of magnitude range (Hell, 2003). This makes transitions between long-lived states very interesting, of course. Here in the equation [Fig. 15(d)], I_s goes down and with that of course also I goes down because one does not need as many photons per second in order to achieve the same resolution d.

The cis-trans isomerization is particularly interesting because it is found in switchable fluorescent proteins. We looked into this very early on, starting from 2003, to check whether we can use it for a STED-like recording. Eventually, I called it RESOLFT, for "reversible saturable/switchable optically linear (fluorescence) transitions" (Hell, 2003; Hell, Dyba, and Jakobs, 2004; Hofmann *et al.*, 2005; Grotjohann *et al.*, 2011) simply because I could not have called it STED anymore. There is no stimulated emission in there, which is why I had to give it a different name. The strength is not only that one can obtain high resolution at low light levels. Notably, one can use inexpensive lasers, continuous wave (CW) lasers, and/or spread out the light over a large field of view, because one does not need such intense light to switch the molecules. In this way, one can parallelize the recordings, meaning that one can make an array of many holes (intensity minima, zeros) at the same time and read out a large field of view quickly (Fig. 16). It does not matter that one has many of these intensity minima at the same time. As long as they are each farther apart than Abbe's diffraction barrier, they can be read out simultaneously by projecting the signal generated in this array of minima onto a camera. Only a few scanning steps in one direction and in the orthogonal direction, and a super-resolution image of a large field of view is taken. In Fig. 17 (Chmyrov *et al.*, 2013) a living cell was recorded within two seconds with more than 100 000 "doughnuts," so to speak, in parallel.

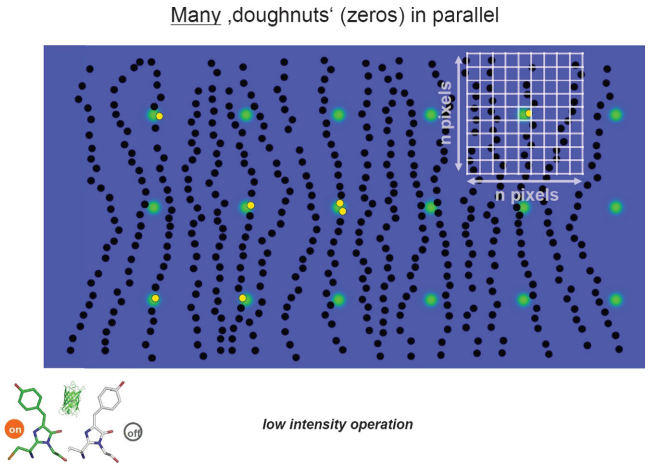

FIG. 16 (color). Parallelization of the STED/RESOLFT concept holds the key to faster imaging. The diffraction problem has to be addressed only for molecules residing within a diffraction-limited region. Thus, many intensity minima ("doughnuts") are produced, at mutual distances greater than the diffraction limit, for highly efficient scanning of large sample areas. The use of highly parallelized schemes is greatly facilitated by harnessing transitions between long-lived molecular on-off states, such as cis/trans.

Notwithstanding the somewhat different optical arrangement, the key is the molecular transition. Selecting the right molecular transition determines the parameters of imaging. The imaging performance, including the resolution and the contrast level, as well as other factors, is actually determined by the molecular transition chosen (Hell, 2009b).

Putting up the next question, what does it take to achieve the best resolution? Now let us assume one had asked this question in the 20th century. What would have been the answer? Well, the answer was unquestionably: good lenses (Born and Wolf, 2002). Sure, good lenses. Why? Because the separation of neighboring features was performed by the *focusing of light*. And then, of course, one needs good lenses to produce the sharpest focal spot of light at the sample here,

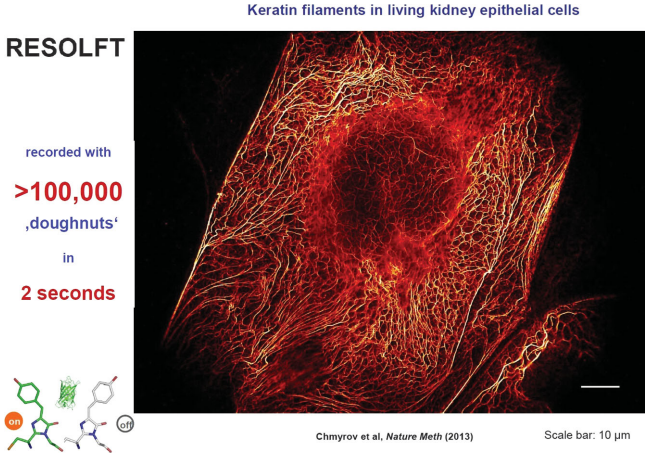

FIG. 17 (color). Massively parallelized RESOLFT nanoscopy. Here, an array of ~114 000 intensity minima (zeros) was used to image a living cell in 2 s.

there, and everywhere, and/ or the sharpest focal spot of light anywhere at the detector. However, once one cannot produce an even smaller focal spot of light, this strategy has come to an end (Fig. 18, top). Therefore, if one has several features falling within a diffraction-limited spot of light, one simply cannot do any better. Resolution is definitely limited by diffraction if one separates features by the focusing of light—no way to tell features, the molecules, apart, because everything overlaps on the detector (Fig. 18, top). So what was the solution to this problem?

Do not separate just by focusing. Separate by molecular states, in the easiest case by on/off states (Hell, 2003, 2004, 2007, 2009a). If separating by molecular states, one can indeed distinguish the features, one can tell the molecules apart even though they reside within the region dictated by diffraction. We can tell, for instance, one molecule apart from its neighbors and discern it (Fig. 18, bottom). For this purpose, we have our choice of states that I have introduced already (Fig. 15), which we can use to distinguish features within the diffraction region.

In the methods I have described, STED, RESOLFT, and so on, the position of the state—where the molecule is on, where the molecule is off—is determined by a pattern of light featuring one or more intensity zeros, for example, a doughnut. This light pattern clearly determines where the molecule has to be on and where it has to be off. The coordinates X, Y, and Z are tightly controlled by the incident pattern of light and the position(s) of its zero(s). Moving the pattern to the next position X, Y, or Z—one knows the position of the occurrence of the on and off states already. One does not necessarily require many *detected* photons from the on-state molecules, because the detected photons are merely indicators of the presence of a feature. The occurrence of the state and its location is fully determined by the incident light pattern.

Now the question comes up: How does this compare with the seminal invention first reported by Eric Betzig (Betzig *et al.*, 2006) based on the discovery of W. E. Moerner (Moerner and Kador, 1989; Dickson *et al.*, 1997) that you can detect single molecules? In the PALM ("photoactivated localization microscopy") (Betzig *et al.*, 2006) concept [also called STORM or FPALM (Hess, Girirajan, and Mason, 2006; Rust, Bates, and Zhuang, 2006)], there are two fundamental differences to STED-like approaches (Fig. 19). First of all, it critically relies on the detection of single molecules. Secondly, unlike in the STED case, in the PALM case the spatial position of the on state is uncontrolled, totally stochastic. A molecule "pops up" somewhere randomly in space, a single molecule per diffraction-sized region, and it is in this way that the on/off state difference is created. But since one does not know where a molecule has turned to the on state, a *pattern of light* must be used with which one can measure the position. This pattern of

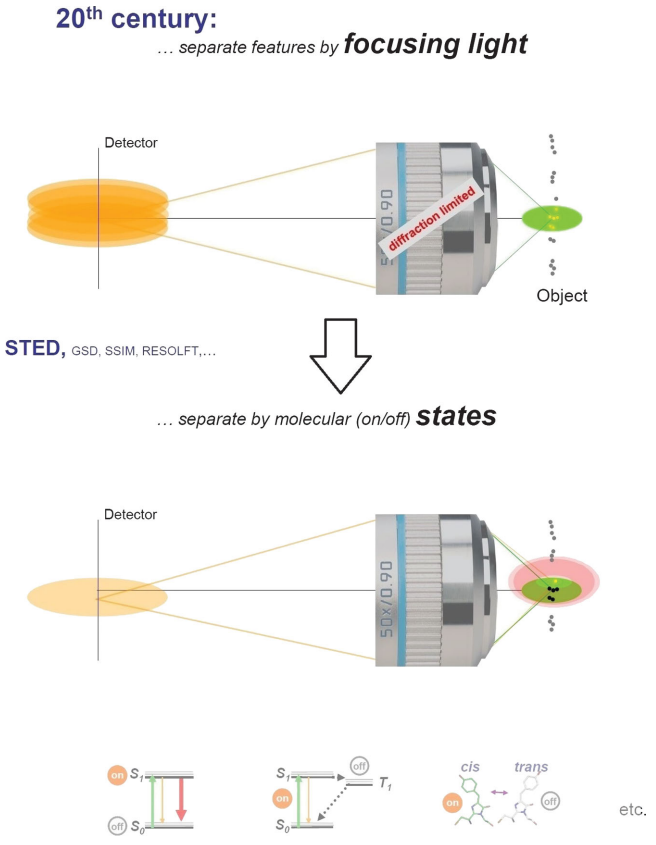

FIG. 18 (color). Paradigm shift in the use of the physical phenomenon by which features are discerned in a far-field optical (fluorescence) microscope: from focusing of light, which is inherently diffraction limited, to using a molecular state transition, such as a transition between an "on" and an "off" state, which is not.

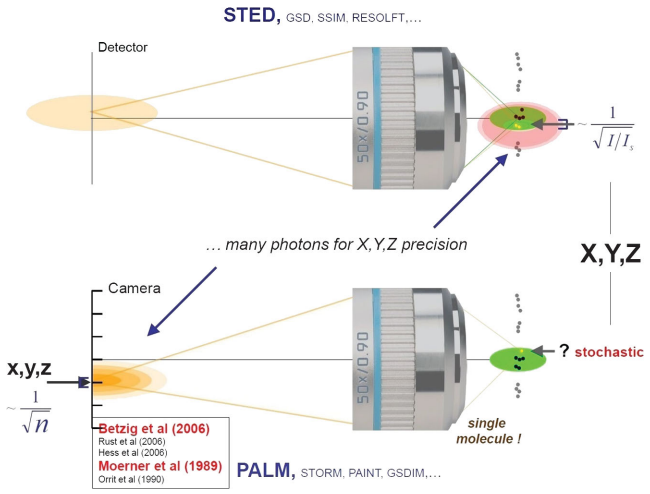

FIG. 19 (color). Both in coordinate-targeted and in coordinate-stochastic nanoscopy methods, many photons are required to define or establish, respectively, molecular coordinates at subdiffraction scales. In the coordinate-targeted mode (STED, RESOLFT, etc.), the coordinates of (e.g.) the "on" state are established by illuminating the sample with a pattern of light featuring an intensity zero; the location of the zero and the pattern intensity define the coordinates with subdiffraction precision. In the coordinate-stochastic mode (PALM, STORM, etc.), the coordinates of the randomly emerging "on"-state molecules are established by analyzing the light patterns emitted by the molecules (localization). Precision of the spatial coordinate increases in both cases with the number of photons in the patterns, i.e. by the intensity of the pattern. In both families of methods, neighboring molecules are discerned by transiently creating different molecular states in the sample.

light is the fluorescent light which is emitted by the molecule and imaged onto an array detector, usually a camera. The pixels of the camera provide the coordinate reference. Without going into the details, this pattern of emitted fluorescence light allows one to determine the molecule's position with a centroid calculation.

An interesting insight here is that one needs a *bright* pattern of emitted light to *find out* the position of emission just as one needs a bright pattern of incident light in STED/RESOLFT to *determine* the position of emission. Not surprisingly, one *always* needs bright patterns of light when it comes to positions, because if one has just a single photon, this goes astray. The photon can go anywhere within the realm of diffraction, there is no way to control where it goes within the diffraction zone. In other words, when dealing with positions, one needs *many* photons by definition, because this is inherent to diffraction. Many photons are required for defining positions of on- and off-state molecules in STED/RESOLFT microscopy, just as many photons are required to find out the position of on-state molecules in the stochastic method PALM.

One is not confined to using a single doughnut (a single diffraction zone) in STED/RESOLFT. We can use a "wide-field" arrangement, meaning that we can also record a large field of view (compare the blue pattern in Fig. 16). To this end, we parallelize the scanning using an array of intensity minima, such as an array of doughnuts. Again, the fundamental difference to the spatially stochastic methods is (Fig. 20) that the positions where the molecules can assume the on or the off state are tightly controlled by the pattern of light with which we illuminate the sample. This is regardless of whether there is one molecule at the intensity minimum of the pattern, or three molecules; however many, it does not matter.

Although the PALM principle can also be implemented on a single diffraction zone only (i.e., using a single focused beam of light), it is usually implemented in a "parallelized" way, i.e., on a larger field of view containing many diffraction zones. PALM parallelization requires that there may be only a single on-state molecule within a diffraction zone, i.e., within the distance dictated by the diffraction barrier. However, the position of this molecule is completely random. Therefore, we have to make sure that the on-state molecules are farther apart from each other than the diffraction barrier, so that they are still identifiable as separate molecules. While in STED/RESOLFT the position of a certain state is given by the pattern of light falling on the sample, the position in PALM is established from the pattern of (fluorescence) light coming out of the sample.

What does I/I_s in STED/RESOLFT stand for? I_s can be seen as the number of photons that one needs to ensure that there is at least one photon interacting with the molecule, pushing it from one state to the other in order to create the required difference in molecular states. I/I_s is, so to speak, the number of photons which really "can do something" at the molecule while most of the others just "pass by." Similarly, in the PALM concept, the number of photons n in $1/\sqrt{n}$ is the number of those photons that are detected, i.e., that really contribute to revealing the position of the emitting molecule. In other words, in both concepts, to attain a high coordinate precision, one needs *many* photons that really do something.

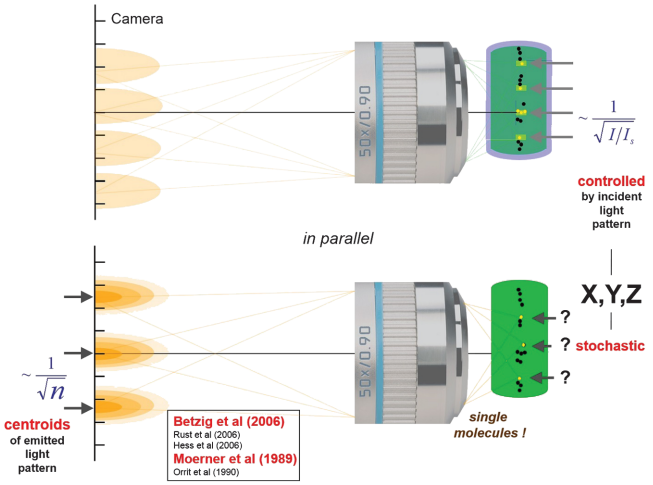

FIG. 20 (color). To parallelize STED/RESOLFT scanning, a "widefield" arrangement with an array of intensity minima (e.g. an array of doughnuts) may be used. The numbers of molecules at these readout target coordinates do not matter, while PALM requires that there may be only a single "on"-state molecule within a diffraction zone, i.e. within the distance dictated by the diffraction barrier. (More precisely: the number of molecules per diffraction zone has to be so low that each molecule is recognized individually.) The position of each on-state molecule is however completely random in space. I_s can be regarded as the number of photons that one needs to ensure that there is at least one photon interacting with the molecule, pushing it from one state to the other in order to create the required difference in molecular states. I/I_s is, so to speak, the number of photons which really elicit the (on/off) state transition at the molecule, while most of the others just "pass by." Similarly, in the PALM concept, the number of photons n in $1/\sqrt{n}$ is the number of those photons that are really detected at the coordinate-giving pixilated detector (camera), i.e. that really contribute to revealing the position of the emitting molecule. In other words, in both concepts, to attain a high coordinate precision, one needs many photons that act.

This analogy very clearly shows the importance of the number of photons to achieve coordinate precision in both concepts.

However, in both cases the separation of features is, of course, accomplished by an on/off transition (Hell, 2003, 2004, 2007, 2009a). This is how we make features distinct, how we tell them apart. As a matter of fact, all the super-resolution methods, which are in place right now and really useful, achieve molecular distinguishability by transiently placing the molecules that are closer together than the diffraction barrier in two different states for the time period in which they are jointly scrutinized by the detector. "Fluorescent" and "nonfluorescent" is the easiest pair of states to play with, and so this is what has worked out so far.

One can take the point of view that in the 20th century it was the lenses which were decisive. And the lens makers ruled the field. One had to go to them and ask them for the best lenses to get the best resolution. But how is it today? No, it is not the lens makers. This resolution game is not about lenses anymore. It is about molecular states, and molecular states are of course about *molecules*. The molecules determine now how well we can image; they determine the spatial resolution. And that is not optical technology—that is *chemistry* (Fig. 21). One

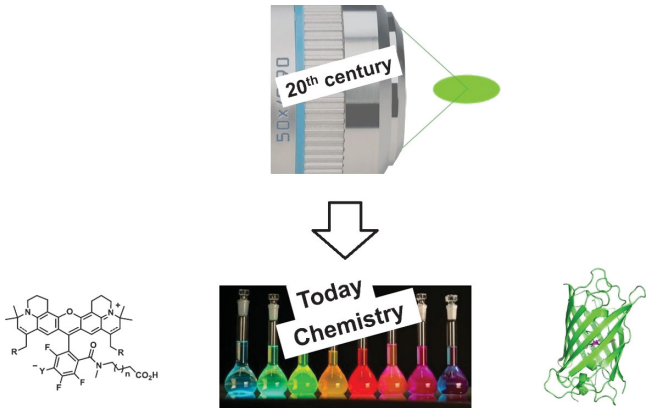

FIG. 21 (color). From lenses to molecular switches. Whereas in the 20th century, the focusing quality of the lenses (i.e. excellent aberration correction) was decisive for gaining the best spatial resolution, now it is the molecules and their state transitions which become central to achieving the best resolution. Optimizing molecules toward providing robust and repeatedly executable (on/off) state transitions (STED/RESOLFT) and photon emissions (PALM) is primarily a chemistry problem.

might say that it is now the chemists who can take the best images. In a way this was initially a physics problem—the diffraction barrier certainly was, no doubt about it—which has now evolved into a chemistry topic.

This Nobel Prize was awarded for superresolution fluorescence imaging. The enabling element being a transition between two states, the two states need not be fluorescence on/off: they could also be a pair of states "A" and "B" (Fig. 22), like "absorption/nonabsorption," "scattering/nonscattering," "spin up/spin down," "bound/unbound" [as in the method called PAINT (Sharonov and Hochstrasser, 2006)], etc. Perhaps one can also imagine a superresolution *absorption* microscope or a superresolution *scattering* microscope, if one identifies the right states. The story continues, and I am expecting more of it to come. It has just begun!

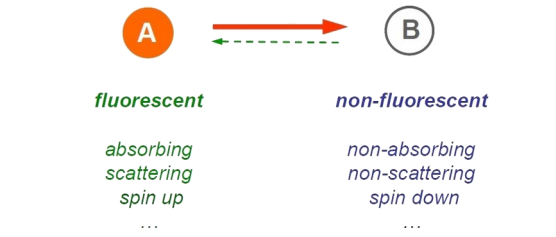

FIG. 22 (color online). The limiting role of diffraction is overcome by utilizing at least two molecular states to separate features residing closer than the diffraction barrier during the brief period in which the features are jointly illuminated for detection. While fluorescent molecules have been the first type of molecules which have provided such states, other molecules and states are conceivable that are not necessarily of the fluorescent type. This is why one could imagine breaking the diffraction barrier also in a nonfluorescence far-field optical microscope, provided suitable states and state transitions are identified.

Looking at Abbe's equation (Fig. 4), it was written in stone for so many years, but it cannot explain the fact that we now have a much higher spatial resolution. Fortunately, we can adapt Abbe's equation very easily. We simply add the square root factor, and now the good news is the resolution goes down to the size of a molecule [Fig. 15(d)]. We can achieve image resolution at the molecular scale.

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I am grateful to Steffen J. Sahl for editing the initial version of the transcribed original lecture, as well as to Mark Bates for further improvements. Last but not least, I would like to mention that I could not have told you about this development without the help of many very talented students and postdocs who have contributed to it. I thank them from the bottom of my heart for their contributions. I would like to add that many of them are still continuing in this field because it is very, very exciting. We thank the Nobel Foundation, Stockholm, for permission to print this lecture. Nobel Lecture, December 8, 2014 (Stockholm University), with an addition from the lecture on December 13, 2014 (Uppsala University).

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