Man the nanoscopes

New light microscopy techniques are pushing the limits of resolution to 50 nm and below. Fluorescence microscopy that rivals electron microscopy in resolution but operates on intact cells may be within reach.

"We've known for 100 years what the limits of light microscopy are," said David Agard (University of California, San Francisco [UCSF], CA) at the beginning of his talk at the American Society for Cell Biology meeting (San Francisco, CA; December 13–17, 2003). "How can we go beyond those limits?" By the end of his talk Agard had answered his own question with a shocking conclusion. "There are," he said, "no fundamental limits to how far we can go."

As proof, Agard showed pictures of a lot of very small polystyrene beads. That they were not only very small but also visible at a resolution of 46 nm was impressive.

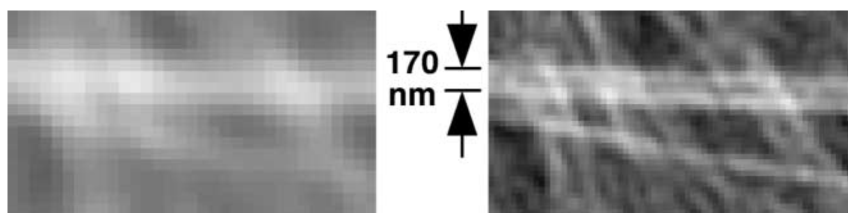
But he also showed a yeast chromosome. Just a blur in the conventional microscopy image, the chromosome viewed by the new techniques revealed perplexing and exciting folds and convolutions. Real biology, Agard seemed to be saying, here we come.

Theoretical limits

The limits to which Agard was referring were first enunciated by Ernst Abbe in 1873. Visible light will only be diffracted by objects above a certain size, said Abbe, just as ocean waves can be diffracted by a row of boulders but not by a row of pebbles. Theoretically, said Abbe, smaller objects cannot be resolved using visible light (Fig. 1). For the current generation of light microscopes that limit has been reached at around 200 nm in the lateral (x,y) direction and 600 nm in the axial (z) direction.

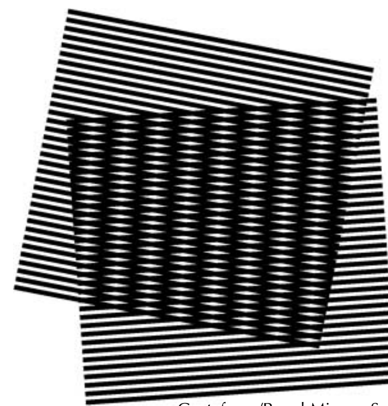
"This resolution issue has been ignored for a long time," says Stefan Hell, a physicist at the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany. "People have said there will be no real chance to do anything about it. I think the current possibilities of fundamentally overcoming a century old limit has surprised many people." Hell withstood some criticism when he claimed, beginning in the mid-1990s, to have broken Abbe's barrier.

Agard presented three techniques developed by collaborating UCSF groups led by John Sedat, Mats Gustafsson, and himself (Gustafsson, 1999). Hell has established conceptually similar techniques, but rather than taking pictures of an entire field of view at once (widefield) he uses confocal systems that scan a sample using a tightly focused beam (Hell, 2003). Other groups working in the field include those led by Andreas Stemmer (Swiss Federal Institute of Technology, Zurich, Switzerland) and Peter So (Massachusetts Institute of Technology, Cambridge, MA).



Gustafsson/Royal Micros. Soc.

Figure 1. **Higher resolution allows two objects to be differentiated, as in the structured illumination image of actin on the right.**



Gustafsson/Royal Micros. Soc.

Figure 2. **A moiré pattern has wider spacing than the two patterns that built it, allowing detection of finer original patterns.**

Adding structure

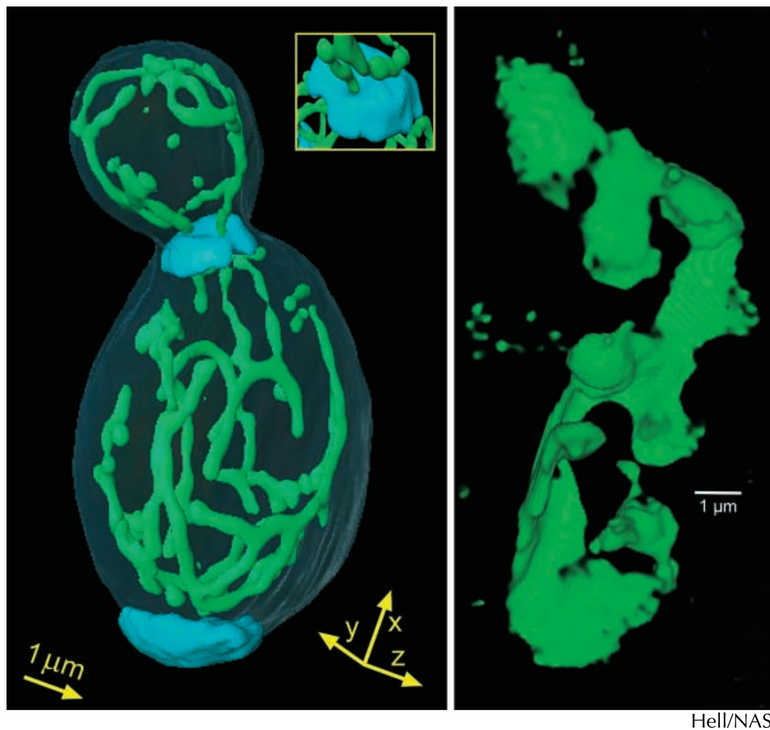
In the first technique, called structured illumination, a diffraction grating is placed in the path of the excitation light. This creates a diffraction pattern on the sample. This fine pattern of light interacts with fine patterns in the sample and creates a moiré effect—an interference pattern whose periodicity is much greater than that of the original pattern (Fig. 2). Thus, fine patterns that were previously below the Abbe limit can now be visualized as a moiré version of their former selves. If the grating is rotated and translated to give 3 different moiré patterns, the original fine pattern can be determined computationally (Gustafsson, 2000).

The pattern from the grating can interact with patterns finer than Abbe's limit, but the illuminating pattern itself is restricted by Abbe's limit. The result is that resolution can be improved by this technique by twofold at best.

© The Rockefeller University Press, 0021-9525/2004/02/337/4 $8.00
The Journal of Cell Biology, Volume 164, Number 3, February 2, 2004 337–340
http://www.jcb.org/cgi/doi/10.1083/jcb.200401065

The Journal of Cell Biology

Downloaded from www.jcb.org on September 30, 2006



Hell/NAS

Figure 3. **4Pi gives a view of yeast mitochondria (left) and mammalian Golgi (right) at ∼100-nm resolution.**

Structure in z

This form of structured illumination is applied by the UCSF team in a lateral (x,y) direction. But structured illumination was arguably first applied by Frederick Lanni (Carnegie Mellon University, Pittsburgh, PA) in the axial, or z direction (Bailey et al., 1993). He shined light through his sample and then had it bounce back from a mirror to interfere with itself. The result was a standing wave that had a periodicity half that of the original wave and was thus able to differentiate features spaced more closely together.

But the planar nature of Lanni's technique was its fatal flaw. The observer could say that a feature was in a peak or valley of the standing wave, but not which peak or valley. Thus, his technique only worked for super-thin samples—those that were as thin as a single standing wave.

While Lanni was developing these ideas, Sedat and Gustafsson were finding similar inspiration but in a less likely arena. "We started attending astronomy meetings in a big way," says Sedat. "That's where a lot of the interferometry started."

Astronomers (and Lanni) use interference between two wave fronts to create more tightly spaced patterns that yield more information. The UCSF and Max Planck groups gave Lanni's technique a twist by relying not on a mirror reflecting planar waves but on focused light provided and observed by lenses on both sides of the sample.

The two-lens solution provides an immediate advantage: emitted light can be harvested from a greater proportion of the sphere surrounding a sample. (Hell's name for his technique, 4Pi, is a reference to $4\pi r^2$ (the surface of a sphere) and the attempt to visualize a sample from all angles.) This reduces but does not eliminate the difference in resolution capability between the x,y axis (comparatively good) and the z axis (usually bad, because light is not being collected around the entire z axis).

The first interference effect with these set-ups is between the two excitation light sources—thus the name incoherent interference illumination microscopy (I^3M) for Gustafsson's version. As it comes from both objectives, the light interferes constructively at the focal plane. But move one quarter of a wavelength away from the focal plane and the two light sources will be half a wavelength out of sync, and thus cancel each other out. The result is a tighter concentration of the effective excitation light at the focal plane (Fig. 3).

In Lanni's technique, these concentrated waves roll away forever as a simple sine wave. But the two light sources used in the new techniques create a series of standing waves whose periodicity varies with the angle of incidence. In the focal plane they all add constructively, but further away they get progressively out of phase and jumbled. This allows both techniques to determine that a feature is at the focal plane and not several wavelengths further away (where the effective illumination is much less).

Gustafsson extends his technique by collecting the emitted light through both objectives and then constructively combining the two beams on a single detector. This image interference microscopy (I^2M) again sharpens the pattern, and the two techniques can be used together as I^5M. The result with both 4Pi and I^5M is ∼7-fold improvement in resolution in the z axis, providing better axial than lateral resolution (Hell and Stelzer, 1992; Gustafsson et al., 1999; Egner et al., 2002). Finally, the UCSF group has combined the two-lens approach with structured illumination to provide resolution that is equal in all directions.

No to linearity

As impressive as these results may be, it is nonlinear methods that could show the most spectacular results in the long term. The key is a phenomenon called saturation. The saturation methods include stimulated emission depletion (STED) microscopy developed by Hell (Klar et al., 2000; Dyba and Hell, 2002), and saturated structured illumination microscopy (SSIM) implemented by Gustafsson, based on theory from Rainer Heintzmann (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany) (Heintzmann et al., 2002).

"It's clear that with a nonlinear phenomenon you can break Abbe's barrier," says Hell. "But common thinking was that nonlinear phenomena would require unacceptably large light intensities."

An example of nonlinearity is multiphoton microscopy, where a fluorophore

Downloaded from www.jcb.org on September 30, 2006

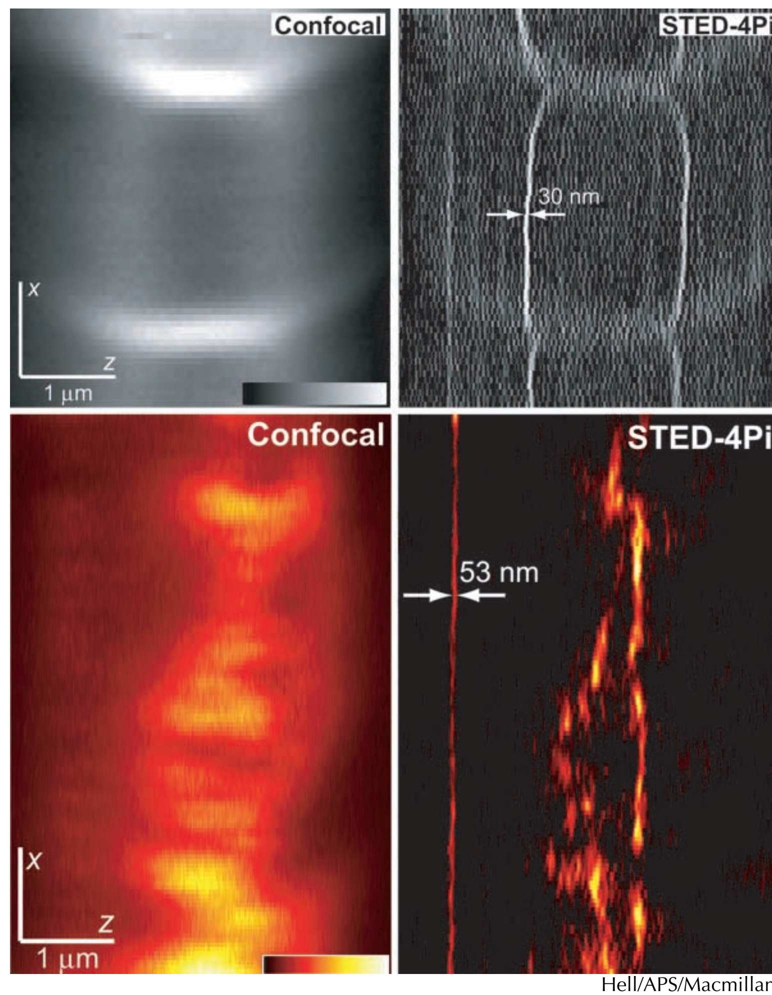
is only excited when it receives hits from two or more independent photons. This results in excitation in only the central, most intense part of the illumination. But if standard fluorophores are used, the energy of the excitation light must be halved, thus doubling the size of the initial light spot. You are lucky if you get back to where you started with the one-photon setup. Multiphoton microscopy has allowed deeper penetration of tissues because of the longer excitation wavelengths, but it has not solved the resolution problem.

STED microscopy suffers from no such constraints (Fig. 4). In STED, an initial excitation pulse is focused on a spot. The spot is narrowed by a second, donut-shaped pulse that prompts all excited fluorophores in the body of the donut to emit (this is the "emission depletion" part of STED). This leaves only the hole of the donut in an excited state, and only this narrow hole is detected as an emitted fluorescence.

The donut idea sounds ingenious but, says Hell, "that's not the trick. The light doing the turning off is also diffraction limited," and so it cannot in theory provide any greater resolution than the original spot. "The trick is that we saturate the depletion—we use enough photons," he says. "This helps me to squeeze the spot down to a very small scale—in principle infinitely."

The nonlinear saturation effect makes the donut look less like a donut and more like the top of a castle turret. The top of the waveform hits a ceiling, the maximum broadens, and the wave squares up. The broadening of the waveform's maximum squeezes the donut hole to be ever smaller, and the squaring gives a more abrupt transition from no excitation to full excitation. Final spot sizes of less than 30 nm have been demonstrated.

The Gustafsson/Heintzmann SSIM is almost the inverse of STED. Now, it is the two interfering illumination sources that are saturating. Together, they form a broad, sharp standing wave. It is the minimum between these waveforms that is narrow, and, once again, the transition from minimum to maximum is more abrupt than normal.



Hell/APS/Macmillan

Figure 4. **Fine features on a bacterial membrane (top) and in a microtubule network (bottom) are visible by STED microscopy (right).**

Whereas Hell makes his excited spot smaller, SSIM makes the excited bands wider. This broadening is what you would expect from saturation, and what makes saturation an undesirable phenomenon for any normal microscopist. Sure enough, says Heintzmann, "the raw data are really ugly. But from this you can calculate a really high resolution image."

Practicalities

To date, much of the literature on these techniques has been in journals specializing in optics and microscopy, and there have been correspondingly few images of interest to biologists. Widespread adoption of the techniques by biologists will have to wait for commercialization of the current designs which, admits Sedat, are "not trivial to use."

Of the new techniques, only a variation of structured illumination is currently part of a commercial microscope. Carl Zeiss has recently started selling the ApoTome as an add-on that does a form of structured illumination. Compared with the UCSF set-up, the ApoTome grating has a wider pattern and does not rotate into different orientations. The result is an improvement in z rather than x,y resolution, resulting in similar performance to a confocal but with the normal widefield advantages (acquisition of the entire field of view at once with a nonlaser light source). This approach was developed by Tony Wilson and colleagues (University of Oxford, Oxford, UK).

The UCSF group has incorporated their full-blown version of structured illumination into their new microscope called OMX, and is talking to companies about commercialization.

Hell says that Leica is developing a compact version of 4Pi. Any double

Downloaded from www.jcb.org on September 30, 2006

objective system such as 4Pi or I^5M must be painstakingly aligned, but Leica has reportedly come up with a system that will resist the battering it may receive from less tech-savvy consumers. Users will still, however, have to get used to the constraints of having lenses on both sides of their samples.

I^5M has trailed 4Pi during development, and does not yet have a known commercial sponsor. But if both systems become available, they will be far from carbon copies. Hell's approach emphasizes solving physical problems by optics. He says his 4Pi system is more robust to sample imperfections because he uses a confocal, 2-photon method that has allowed live imaging. But these methods add complication and expense to the equipment. Gustafsson, by contrast, uses the simpler and potentially faster wide-field microscopy of I^5M and solves more of his problems with computational manipulation post-acquisition.

The nonlinear techniques are even further from widespread application. The original version of STED microscopy requires two extremely expensive lasers capable of firing high energy pulses within picoseconds of each other, although the same effect has recently been demonstrated with laser diode sources. The main challenge for SSIM is the accelerated bleaching that occurs under saturating light intensities.

However the differences in equipment shake out, both teams agree that the constraint of Abbe's limit has been well and truly broken for fluorescence microscopy. Interference and nonlinear effects shape the excitation light within the sample such that it can detect finer details. With this principle established, the practical instruments are sure to follow. "There are a lot of things you can do to improve resolution," says Sedat, "and we're just scratching the surface."

William A. Wells
wellsw@rockefeller.edu

Thanks to Mats Gustafsson and Stefan Hell for their efforts in explaining this work.

References

Bailey, B., D.L. Farkas, D.L. Taylor, F. Lanni. 1993. Enhancement of axial resolution in fluorescence microscopy by standing-wave excitation. *Nature.* 366:44–48.

Dyba, M., and S.W. Hell. 2002. Focal spots of size λ/23 open up far-field fluorescence microscopy at 33 nm axial resolution. *Phys. Rev. Lett.* 88: 163901.

Egner, A., S. Jakobs, S.W. Hell. 2002. Fast 100-nm resolution three-dimensional microscope reveals structural plasticity of mitochondria in live yeast. *Proc. Natl. Acad. Sci. USA.* 99:3370–3375.

Gustafsson, M.G.L. 1999. Extended resolution fluorescence microscopy. *Curr. Opin. Struct. Biol.* 9: 627–634.

Gustafsson, M.G.L, D.A. Agard, J.W. Sedat. 1999. I^5M: 3D widefield light microscopy with better than 100 nm axial resolution. *J. Microsc.* 195:10–16.

Gustafsson, M.G.L. 2000. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *J. Microsc.* 198:82–87.

Heintzmann, R., T.M. Jovin, C. Cremer. 2002. Saturated patterned excitation microscopy-a concept for optical resolution improvement. *J. Opt. Soc. Am. A.* 19:1599–1609.

Hell, S.W. 2003. Toward fluorescence nanoscopy. *Nat. Biotechnol.* 21:1347–1355.

Hell, S.W., and E.H.K. Stelzer. 1992. Properties of a 4Pi-confocal fluorescence microscope. *J. Opt. Soc. Am. A.* 93:277–282.

Klar, T.A., S. Jakobs, M. Dyba, A. Egner, S.W. Hell. 2000. *Proc. Natl. Acad. Sci. USA.* 97:8206–8210.

Downloaded from www.jcb.org on September 30, 2006