

# microScience Imaging

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## Examining the Body's Inner Clock

By Christoph Bauer, *National Center of Competence in Research, University of Geneva, Switzerland*

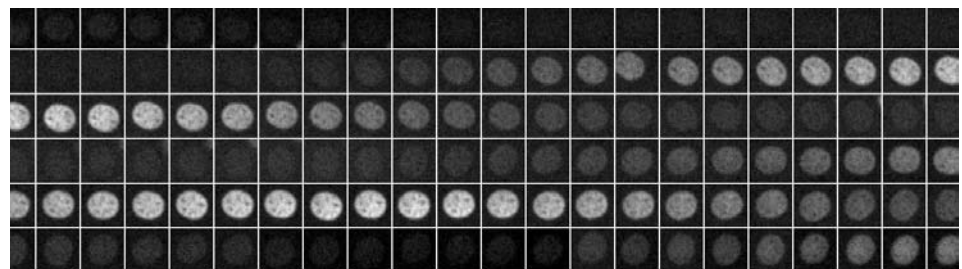
### New perspectives for cancer research and drug development

Almost everyone has experienced jet lag after flying across time zones. Typical symptoms, that include difficulty falling and staying asleep and digestive disturbances, are caused by circadian rhythms, which exist in the brain and other organs. Proteins are synthesized in the body and automatically regulate their own deactivation in a continuous 24-hour cycle. The body's central clock is controlled by daylight. In addition to the sleep/wake cycle and the digestive system, circadian rhythms control blood pressure, body temperature, hormone changes, and libido.

### Rhythms also exist in single cells

In the early 1990's, a research group headed by Professor Ueli Schibler of the University of Geneva, proved the existence of circadian rhythms in cell lines. However, Schibler and his team did not stop there. In 2004, they were the first to prove the existence of rhythmic protein synthesis in single cells. These results opened up new avenues for cancer research, as defects between protein synthesis and deactivation are typical of various forms of cancer.

Proving the existence of circadian rhythms in an individual cell was intensive work. Cells were observed continuously for up to three days. They were treated with a fluorescence marker and a substance to ensure fast degradation of the proteins. Based on the fluorescence profile, research determined that the "inner clock" is also active in individual cells.



Top: Leica AF6000 LX Bottom: The Geneva-based research team captured images every 30 minutes for 3 days.

### Long-lasting stability

"We could not have achieved these outstanding results without the Leica AF6000 LX fluorescence workstation," explains Christoph Bauer, a member of Schibler's research team. A common problem source in time-lapse imaging is thermal effects that cause drifting focus during image capture. The inverted microscope's climate chamber provides constant temperature control. The climate chamber maintains constant CO<sub>2</sub> values and humidity to protect cells. The camera's shutter and illumination are controlled with millisecond precision. "Capturing images in real time gives a decisive advantage," Bauer notes. "The AF6000 LX illuminates only when an image is being captured, which prevents cell bleaching."

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# Technology Fast Track

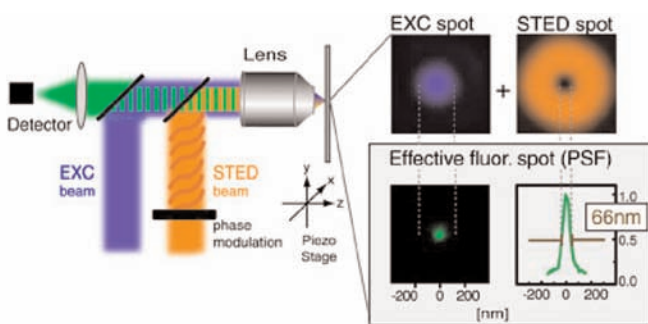
## Shattering the Abbe Limit

By Tobias Jacob, *Leica Director of Technical Support* and Lon Nelson, *Leica Marketing Manager*

For almost 135 years, light microscope imaging has been governed by the diffraction-limited laws established by Ernst Abbe. Today's advanced, laser-based confocal microscope systems can reach this limit, which is ~200nm in both x- and y-axes. This threshold has recently been shattered by Stefan Hell of the Max Planck Institute for Nanobiophotonics in Goettingen, Germany using a technology called STimulated Emission Depletion or STED.

STED technology is used in conjunction with a laser scanning confocal microscope and serves to 'sharpen' the emission spot or Point Spread Function (PSF), resulting in resolution of 90nm or better. This is accomplished via a ring-shaped beam that effectively depletes excited molecules in the outer focal area at the specimen plane. By blocking or depleting fluorescence emission around the focal point, STED increases the sharpness of focus and results in an image that is no longer wavelength or diffraction limited. Rather, the image resolution is only limited by the power of the STED ring and how much power the specimen can tolerate.

How is this accomplished? Two tightly synchronized, superimposed, picosecond-pulsed excitation and emission beams work in concert to create the STED effect while scanning across the field-of-view (FOV). See **Diagram below**.



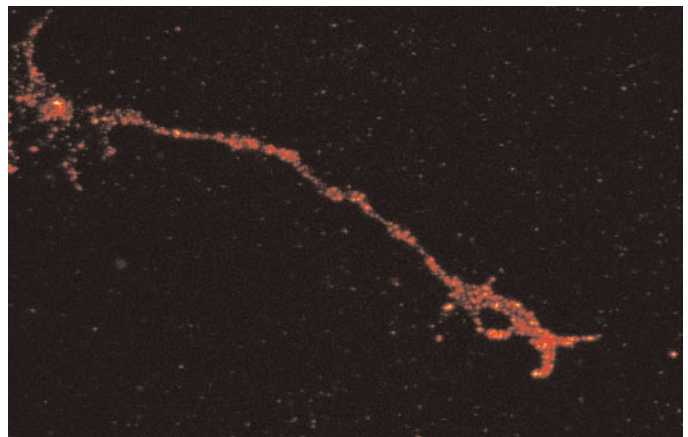
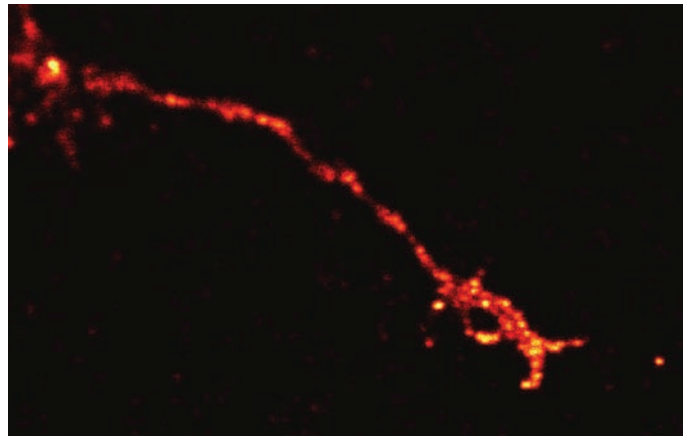
**Diagram:** Courtesy Stefan Hell, MPI for Nanobiophotonics, Goettingen, Germany

Beyond the obvious resolution advantage, STED technology offers other benefits. The same red-shifted (735-800nm) STED depletion laser can be used for standard 2-photon confocal excitation. Up to four standard spectral confocal channels can be employed simultaneously to the super-resolution channel, and STED will be available as an upgrade option for future expansion needs. Further, STED offers automatic laser alignment of both excitation and depletion lasers, and the emission detection can be switched from avalanche

photo detectors for the highest sensitivity to photomultiplier tubes for the largest dynamic range.

Interested scientists should also be aware of limitations with STED technology. Currently STED is designed for use with just 2 fluorescent labels, ATTO 647N and ATTO 655, and is not for use with live cells. Also, the scan field is limited to 50x50 microns square or smaller, pixel size is < 30nm for full resolution transfer, and typical frame rates are approximately 1 minute.

Despite these limitations, STED should quickly prove to be a breakthrough that drives life science research further than ever before possible. Later this year, the first commercially available STED system will be offered by Leica Microsystems on the current Leica SP5 confocal platform.



*Top image – Confocal:* Drosophila neuromuscular synapses, presynaptic active zones (Ca++ channels). *Bottom image – STED:* Bruchpilot protein stained with ATTO 647N. 2048x2048 pixels. Courtesy: Stephan Sigrist, Wuerzburg, Germany.

# The Objective

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## Visualizing Life and Luminous Killer Proteins

By Anja Schue, *Leica P.R. Editor*

Molecular biologists can watch living cells at work with the aid of fluorescence microscopy, and with confocal laser scanning microscopy in particular. Researchers can also view minute, living tools within cells: proteins that fluoresce when excited with laser light.

One of the renowned researchers in the field of fluorescent proteins (FP) is Dr. Konstantin Lukyanov from the Institute for Bioorganic Chemistry of the Russian Academy of Sciences in Moscow. In September 2006, he presented "Novel Fluorescent Proteins for a Deeper Insight into Biology" at the German Cancer Research Center (DKFZ) in Heidelberg as part of the Leica Scientific Forum – Advances in Life Science series. Lukyanov provided an overview of FP research and his current projects.

### A new research standard

The green fluorescent protein of the pacific jellyfish *Aequoria Victoria*, known to scientists simply as GFP, was discovered by Japanese researcher Osamu Shimomura in 1962 and was the first naturally fluorescent protein to be discovered. In 1994, GFP was first used successfully as a marker for other proteins. GFP is inserted into genes at precisely the point responsible for synthesis of the proteins to be studied. The cell then produces the protein together with the GFP. The fluorescence of the GFP makes the temporal and spatial distribution of the target protein visible in the cell. GFP can be used to visualize cells of virtually any life form without adverse side effects, which makes GFP ideal for in-vivo studies of biological processes.

The use of FP is now a standard in biological and medical research, for example when studying gene expression. It is now possible to create proteins related to GFP that fluoresce in other colors such as yellow, blue or red. Various parts of the cell and interactions between proteins can be investigated using a variety of fluorescent markers.

### Promising new variants

Biologists have since discovered other FPs that occur naturally, such as those in coral or sea anemones. For example, Lukyanov isolated an FP from the *Dendronephthya* coral that switches from green to red when excited with light. The original protein, which was very large and unwieldy for analysis purposes, was genetically modified to create a smaller, more versatile variant. The changing fluorescence of this Dendra marker can help improve the understanding of protein synthesis and breakdown in the cell.

Another protein Lukyanov is investigating is known as "KillerRED," a unique phototoxic FP. When KillerRED is excited, it not only fluoresces, it also releases highly toxic reactive oxygen, which can kill living cells within minutes. Such side effects are generally undesirable when studying biological processes. However, Lukyanov has completely different applications in mind. Should it become possible to introduce the KillerRED protein into cancerous cells in a targeted manner, light activation could be used to destroy tumors. Lukyanov and his team are only just beginning their research of phototoxic FP, but the results so far are promising.



## For You

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# The Private Eye

## To See or Not to See

By Rob Kimura, *Leica Product Manager*

As the evolution of digital photomicrography continues, new generations of high-resolution cameras have become available for life science imaging. Digital camera technology is mainly driven by the consumer market where ‘the more pixels the better’ is the status quo. In recent years the consumer market has seen color cameras jump from 1.3 megapixels to 12 megapixels and higher. A common question people ask is, “How many megapixels do I need?” But the real question should be, “What do I gain by using a high-resolution digital camera on my microscope?”

### Understanding image formation

Due to the physics of the image formation process, even a perfect microscope objective will blur two adjacent objects into a single object when placed close enough together (the resolution limit). One way to visualize this ‘limiting resolution’ is to image a repeating pattern of adjacent black and white lines. When the number of ‘line pairs’ per millimeter (lp/mm) is increased beyond the optical resolution limit of the microscope, the image will no longer form lines, but instead will form a uniform gray background.

In addition to offering a specific level of image detail, an objective lens also magnifies an object. At the camera’s digital sensor, this translates into an image spread across a larger area than the chip size whenever magnification to the camera is greater than 1x. To compensate for this discrepancy, cameras are attached to the microscope via a C-mount. The C-mount can have varying magnifications itself to properly format the image to the chip size.

### Perfect pixel use

From the previous discussion of line pairs, one would expect that the ideal pixel correlation would place 2 pixels across each line pair so that one pixel can detect the white line and the other pixel the black line. However, this pixel ratio can produce a gray result because pixels can be placed between the white and black lines, if you imagine moving the specimen laterally. To resolve all line pairs in all cases, there must be at least 3 pixels per line pair. As you can see in the ideal case shown in **Diagram 1**, with at least 3 pixels per line pair, the camera can now detect the line pairs, even if pixels shift to the left or right. It is important to note that further increasing the number of pixels can lead to ‘over-sampling’, where the additional pixels per line pair provide no gain in spatial information. However, the transition to over-sampling depends on the wavelength of light used, the objective’s numerical aperture, magnification to the camera, and the camera’s pixel size.

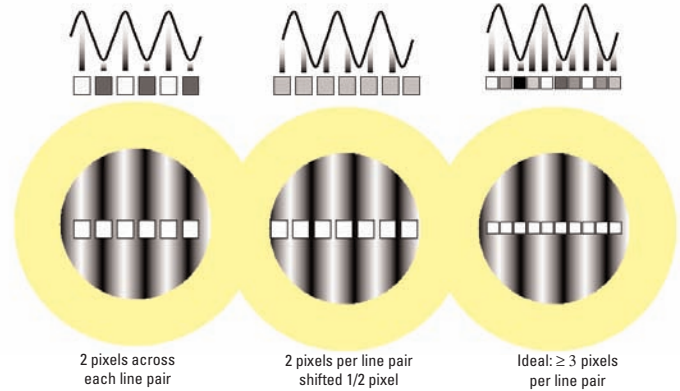


Diagram 1

### Determining resolution as defined by line pairs

With simple assumptions, we can estimate the limiting resolution for a microscope objective, determine the number of line pairs across the field of view (FOV), and compare this to the number of pixels covering the same distance for a given camera. There are many mathematical definitions for optical resolution (R), but a simple approximation to start with is:

$$R = \frac{\lambda}{2(NA)}$$

where  $\lambda$  is the wavelength of the light in nanometers (nm)

This relationship indicates that when using a high NA lens (1.46) and white light illumination (assuming a midpoint of 550nm), the smallest resolvable distance is about 188nm or 0.19 $\mu$ m. To translate micron resolution to line pairs per mm (lp/mm), use the following equation:

$$\frac{1}{(\text{Resolution in mm})} = \text{lp/mm}$$
$$1 / .000188 = 5319 \text{ lp/mm}$$

To calculate the number of line pairs required to cover the objective’s FOV, calculate the area visible through the lens by dividing the FOV of your microscope’s eyepieces (let’s assume 25mm) by the magnification factor.

**A 5x objective allows observation of a 5mm area**  
**A 100x objective allows observation of a 0.25mm area**

So how many line pairs can be observed with a 100x objective? Simply multiply the visible area by the optical resolution in lp/mm to get total line pairs. Please note that there is one caveat in this calculation. A microscope objective forms a circular image, but a camera sensor is typically square or rectangular. If the FOV for the microscope is 25mm, then the FOV of the sensor is reduced by the square root of 2.

*continued on page 5*

## To See or Not to See

(continued from page 4)

**Example: Using a 100x objective with a 1.46 NA and a 25mm FOV**

$$\frac{0.25}{\sqrt{2}} = 0.18\text{mm visible area}$$

**Achievable Optical Resolution = 5319 lp/mm from above**

**Line pairs covering camera sensor = 0.18mm x 5319 lp/mm = 957 lp**

### Using a monochrome camera

The monochrome camera presents an ideal case as every pixel contributes equally to the resolution. The number of pixels required to capture every bit of spatial information coming from the objective is:

**Pixels across digital sensor = 957 lp x 3 pixels/lp = 2871 pixels**

Camera resolution is specified in terms of the total number of pixels, so assuming a rectangular 4:3 format, the number of pixels needed for ideal digital image quality is:

**2871 pixels x [2871 x (3/4)] pixels = 6 megapixels**

### Using a color camera

A pixel in a color (Bayer Matrix) camera performs two functions, spatial sampling of the image and measuring the intensity for a specific color of the spectrum, e.g., red, green, and blue. Consequently, it takes more pixels (by convention, approximately 25% more per line pair) to obtain the same resolution as a monochrome camera. So, if 4 pixels/lp are required for this example:

**3828 pixels x [3828 x (3/4)] pixels = 11 megapixels  
required camera resolution**

Now work through the example in **Diagram 2**, with different starting points. What do you notice about the end result? In **Diagram 2**, with higher magnification, a lower megapixel camera is required. This is counter-intuitive, but true nonetheless.

### Do I need a high-resolution digital camera?

Now let's get back to our original question. High-resolution, megapixel cameras provide a definite resolution benefit at low magnification. But as magnification increases, they provide less of an advantage. What is optimum depends on what kind of imaging you are doing. More pixels will increase the size of the image file. But, more importantly, as the number of pixels increases, each pixel is typically smaller, which results in reduced dynamic range and light sensitivity, which are critical factors in fluorescence imaging.

The bottom line is that higher megapixel cameras make sense for very low magnifications, brightfield imaging typically seen in

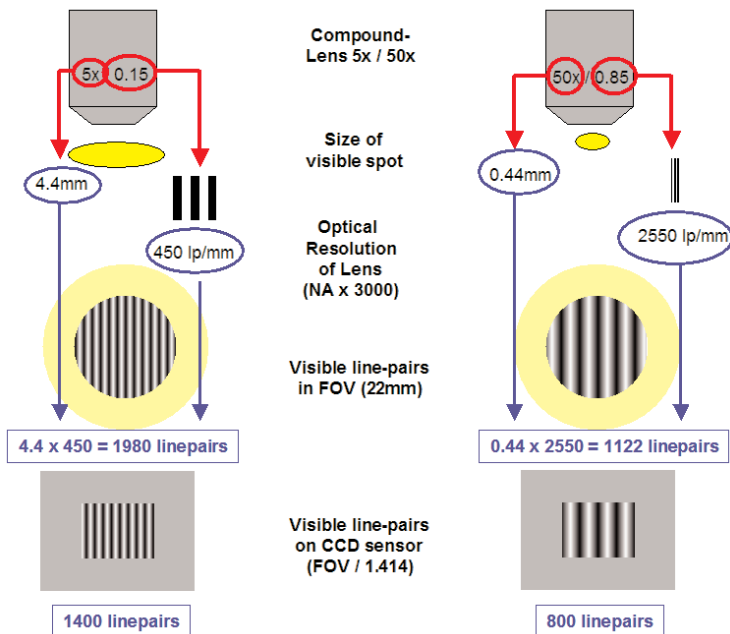


Diagram 2

stereomicroscopes, or if the entire available FOV is used. But they are not necessarily the right solution for high magnification applications, particularly in fluorescence, that require a wide dynamic range and superb light sensitivity.



## Your Educational Resource

### Marine Biological Laboratory

- Analytical & Quantitative Light Microscopy (**May 9-18**)
- Embryology: Concepts & Techniques in Modern Developmental Biology (**June 9-July 22**)
- Neurobiology (**June 2-July 29**)
- Physiology: Modern Cell Biology Using Microscopic, Biochemical, and Computational Approaches (**June 9-July 29**)

<http://www.mbl.edu/education/courses/summer/index.html>

### Cold Spring Harbor Laboratory

- Cell & Developmental Biology of Xenopus (**April 14-24**)
- Molecular Embryology of the Mouse (**June 6- 26**)
- Molecular Techniques in Plant Science (**June 29-July 19**)

<http://meetings.cshl.edu/courses.html>

### University of British Columbia

- 3D Microscopy of Living Cells (**June 16-28**)

<http://www.3dcourse.ubc.ca>

# Open Forum

## What is a White Light Laser and How Does It Work?

By Lon Nelson, *Leica Marketing Manager*

Most of today's confocal systems merge multiple laser lines in order to provide specific excitation for various fluorescent labels from blue to red. While these systems provide gorgeous images and serve to advance scientific research, the use of multiple, large laser sources has become more and more expensive, occupies a lot of valuable lab real estate, produces a significant heat load, and ends up being inflexible with the amount of different fluorescence labels that come through a core imaging facility.

A remedy for all these ills is a single illumination source that covers the entire visible spectrum and does not limit imaging performance ... a white light laser. Later this year, Leica will introduce such a laser. With a continuous spectrum in the visible range, the white light laser will be freely tunable, which will make it the most flexible laser source on the market. Simultaneous intensity and wavelength control will be available for eight separate lines, and when a new fluorescent probe becomes commonplace, there is no need to purchase another laser as the white light laser can simply dial-in to the required excitation wavelength.

At the heart of the white light laser is a 1060nm picosecond-pulsed laser, which illuminates a special fiber with a specific core structure. By an intricate non-linear process, different colors of the visible spectrum can be produced. Based on feedback from the microscope imaging system, this full spectrum can then be freely tuned to exact wavelengths via an AOBs (acoustic optical beamsplitter) and supercontinuum fiber.

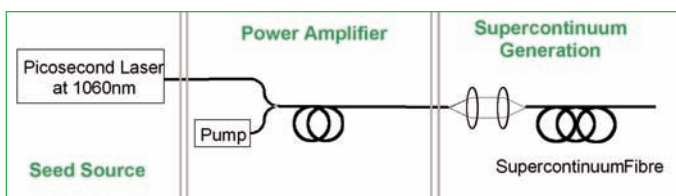


Diagram 1: Basic design of Leica's white light laser.

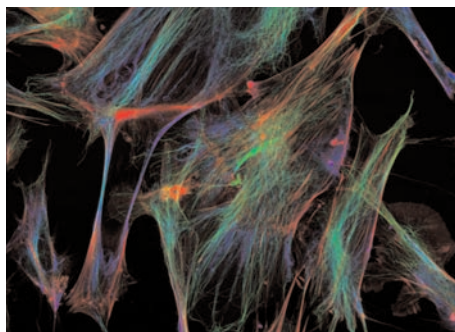


Diagram 2: Mouse fibroblasts, triple stained imaged with white light laser. Blue: Microtubuli, Green: F-Actin, Red: Vimentin.

# Upcoming Events

Visit Leica Microsystems at the following exhibitions:

- The G.O.T. Summit 2007, Boston, MA **April 12 -13, 2007**
- American Association for Cancer Research (AACR) 2007, Los Angeles, CA **April 15 -18, 2007**
- Experimental Biology (FASEB) 2007, Washington D.C. **April 29 - May 1, 2007**

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*Note: We are interested in your comments and thoughts about the newsletter. Please feel free to email your comments to: [microscience.imaging@leica-microsystems.com](mailto:microscience.imaging@leica-microsystems.com)*