1 iChrome: The new ultrachrome light source from Toptica

New ultrachrome light source for microscopy

CONTINUOUSLY TUNABLE COLOR LASERS OFFER MAXIMUM FLEXIBILITY

Wavelength flexibility and spectral purity are decisive factors in modern biophotonics. Various new laser concepts such as super-continuum (or white light) lasers currently cover a wide range of wavelengths in the visible spectrum. However, these have limited spectral purity and also have a relatively low effective efficiency.

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A totally new short pulse fiber based laser concept, the ›iChrome‹, provides both continuous wavelength coverage from blue to red and, due to its design principle, an optimum emission free from background. In conjunction with the pulse shape, it is an ideal laser source for a range of biophotonic applications.

For example, in cell biology fluorescence microscopy has advanced to become a standard technique in the last two decades. The two main reasons for this are its selectivity and the high contrast provided by the imaging method. The variety of different fluorescence markers that have been developed in this time is equally important. Currently, these can be selectively chemically bonded to almost any molecule in a cell, thus allowing targeted examination of those same molecules. In contrast to other imaging methods, the contrast in fluorescence microscopy is so high that even individual modules can be detected. This capability is in part made possible by the ›Stokes shift‹ that moves the fluorescence into the red compared to the excitation light. Spectral filters allow the excitation light to be masked, providing a detection free of background and an exceptionally high contrast.

Fluorescence markers

The specific tagging of the cells with multiple dyes resulted in a major breakthrough in clinical applications. Here, so-called screening methods are used, whereby the reaction in a large number of cells is examined in order to indicate, for example, the effectiveness of drugs. Only statistical analysis of large data volumes helps scientists to arrange the natural fluctuations in the measured variables for living cells into a consistent picture of reaction paths.

In addition, the use of multiple dyes enables data acquisition to be automated. One dye marks the cell nucleus and is only used to identify the number of cells examined. A further dye only begins to fluoresce once the desired reaction occurs. A third dye is used as a so-called ›counter stain‹ and its purpose is to act as a control group in the experiment. Digital camera images of a large number of cells are automatically analyzed using an algorithm during screening. This identifies the cell nuclei and determines the integral intensities of the other two dyes. This enables a measure to be defined for how well the reaction under investigation is proceeding in the cells: The higher the intensity of the reaction dye (measured by the number of cells against the intensity of the control dye), the more effective the drug.

Excitation light sources

This simple example clearly highlights the requirements for the light source in fluorescence microscopy. It must selectively excite the wide range of dyes and additionally be easy to integrate into an automated environment.

Xenon, mercury or halogen lamps have traditionally been used in the past,

making use of an appropriate filter to select the required wavelength range for excitation. Because of the relatively short service life of these lamps, however, modern microscopes are increasingly switching to LEDs. Their major advantages include their durability and value for money, although they are not as broadband as lamp sources and also cannot be tuned in wavelength. A separate, additional LED with an optimized spectrum is thus needed for each dye. This limitation leads to multiple LEDs being combined in a single lamp unit and their light being overlaid by beam splitters before being guided into the microscope. This cascading of multiple sources has been the only method available for the excitation of multiple dyes.

One thing that both conventional and modern light sources have in common is that their luminosity is only sufficient for wide-field microscopy. That is, the light source illuminates a broad area uniformly and this area is imaged by the microscope. As a result, the depth of focus is not particularly high, resulting in fluorescence originating outside the focal plane contributing to a fuzzy background in the image.

Confocal microscopy provides a remedy here, where the field of view is scanned one point at a time and the undesirable light components are filtered out with a pinhole aperture. The gain in depth of focus enables individual optical sections of the entire cell to be recorded and these can then be reconstructed in three dimensions on a computer. With this technique, not only can the overall behavior of cells be studied, but also processes within the cells themselves. For the case of a tested drug, it is possible to identify exactly where in the cell its effect occurs. This allows targeted improvements to be made, for example in terms of absorption into or reactions with particular components of the cell.

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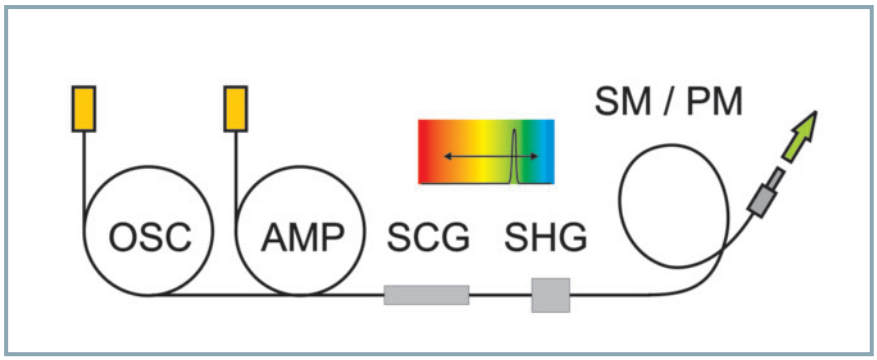


2 iChrome functional principle. OSC/AMP: fiber laser, SCG: super-continuum in IR, SHG: frequency doubling into visible range, SM/PM single-mode, polarization maintaining light fibers

The main reason why confocal microscopy has not become established in all laboratories is primarily due to the light source. Because of the low transmission of the pinhole aperture, high powered lasers need to be used, although such lasers were previously not easily available and were very expensive. Again, as the bandwidth they provide is significantly narrower even than LEDs, several different lasers have to be combined if multiple dyes are to be excited. All of this makes the method expensive and complex.

Widely tunable light sources – ›ultrachrome‹

With the advent of widely tunable light sources, there is now a solution for this dilemma. The term ›ultrachrome‹ refers to a high powered laser whose wavelength can be tuned over the entire visible spectrum.

For widespread use in cell biology, easy handling and automatic tuning are also essential. This essentially limits the choice of ultrachrome light sources to two versions: super-continuum white light sources and a new frequency conversion approach realized by Toptica.

A typical white light source consists of a pulsed laser with a power of several watts, the pulses triggering a variety of non-linear processes in a wave guide. The resulting emission spectrum is known as a super-continuum, as it covers the spectral range of the conventional light sources mentioned above. One consequence of this effect is that the spectral coherence of the laser is lost, meaning that the white light source can effectively be viewed as a simple lamp with a much higher luminance.

This luminance does not refer to the intensity, which is typically only a few milliwatt per nanometer, but rather to the spatial coherence. The light is emitted from a very small area, the fiber, and all of the light can then be focused onto a small area of the specimen. The luminance is thus many times higher, high enough to allow confocal imaging.

The advantage of white light sources is their simple construction with just two components – the laser and the fiber. However, the approach of utilizing the non-linear processes in the fiber conceals a major disadvantage. These processes are very sensitive and they also influence one another, meaning that they are very difficult to control and leading to a loss of spectral coherence, respectively. ▶

The noise (intensity and polarization) of such sources cannot normally be ignored.

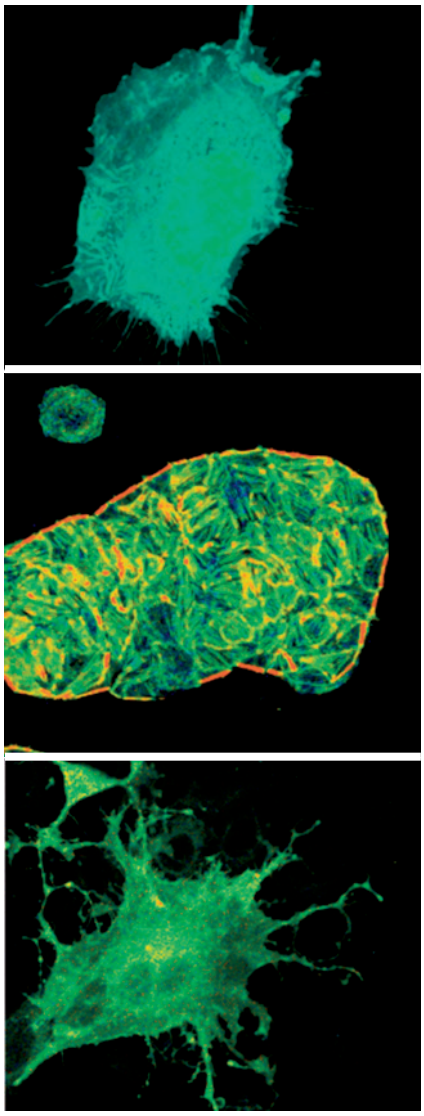
A new approach

With the ›iChrome‹, Toptica has developed a different type of ultrachrome light source based on frequency conversion (Figure 1). Its core is an erbium-doped fiber laser, which generates a near infrared frequency component in a highly non-linear fiber. This design is very similar to that of the white light source but has a crucial difference – the spectral coherence is retained. The reason for this is that only the first step of super-continuum generation is carried out and the unmanageable variety of subsequent processes is suppressed. However, this first step can be controlled very effectively using the pulse duration of the fiber laser. This results in targeted spectral tuning of the infrared frequency component. A second step then efficiently doubles the frequency of the near infrared coherent light, providing wavelengths of 488 to 640 nm for microscopy.

This approach is more complex (Figure 2) as it consists of three components – laser, fiber and frequency doubling. However, this division means that the tunable laser light can be more effectively controlled at each individual step, leading to lower noise and retention of the spectral coherence. The laser also has significantly lower output power, as it does not have to drive a large number of non-linear processes. The power consumption is so low that the unit can be operated without cooling, which is normally associated with interfering noise.

In contrast to the super-continuum, only a very narrow band, spectrally pure laser line is generated. This has both advantages and disadvantages. For example, only one dye can be excited per image, although the rapid automated tuning ensures that successive images of different dyes can be recorded, thus making this disadvantage effectively irrelevant in practice.

The key advantage of this approach strongly favors high contrast imaging in fluorescence microscopy: the individual laser line does not overlap with the fluorescence and the residual laser light at the spectral position of the fluorescence is at least six orders of magnitude weaker. By contrast, a white light source emits over the entire spectral range and must be suppressed using filters, meaning that the contrast can suffer under unfavorable circumstances.



3 FLIM image of Cos7 cells dyed with green/yellow and recorded at 487 nm. Fluorescence lifetime: 2.4 ns
4 FLIM image of HT29 cells dyed with Atto532 and recorded at 535 nm. Fluorescence lifetime: 2.1 ns
5 FLIM image of Cos7 cells dyed with DsRed monomer and recorded at 547 nm. Fluorescence lifetime: 1.5 ns
(Images Dr. Nitschke, by kind permission of Carl Zeiss MicroImaging)

Application in FLIM

An example application – the well established fluorescence lifetime imaging microscopy, or FLIM – highlights the numerous advantages of ultrachrome laser sources. In this technique, pulsed excitation is used to generate fluorescence of dyes in the specimen. The fluorescence decay rate, or the related fluorescence lifetime, averaged over a large number of pulses, is determined at each point in the image (Figures 3, 4 and 5). Each color represents a different lifetime. FLIM thus adds a fourth dimension to three-dimensional space resolution, namely time resolution. This makes the dye the smallest imaginable reporter in cell biology, providing valuable 4D information.

The use of an ultrachrome light source enabled each dye in the images to be excited at its maximum excitation (487, 535 and 547 nm), thus minimum laser intensity could be used in order to avoid cell damage. Unlike pulses from conventional laser diodes, the iChrome laser pulses exhibit no undesirable post-pulse artifacts. This, coupled with the low noise performance, ensure that the confocal FLIM images are of exceptionally high quality.

Summary: optimum flexibility with ultrachrome lasers

Ultrachrome light sources will be a key element of future laser development for a range of biophotonic applications. As well as providing a free choice of wavelength in the visible range, spatial and spectral purity as well as the ideal short pulse dynamics open up various applications. The brand new iChrome laser concept differs from conventional super-continuum laser sources, particularly in terms of the improved noise behavior and retention of spectral coherence.

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