

Industrial Diode Lasers

Exploiting diode lasers for confocal microscopy

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The battle against diseases of our time

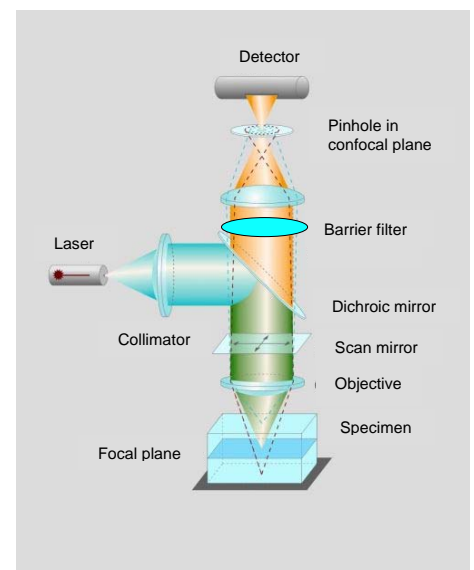
Modern medicine offers successful treatment of various illnesses. However, there is no total cure available for diseases such as Alzheimer, Parkinson or AIDS. The development of a suitable agent requires total understanding of all involved biological processes. Thus researchers need to take a look at the cellular level of human tissue and watch the production of proteins, exchange of hormones as well as the varying content of specific ions.

Microscopes – great tools for research

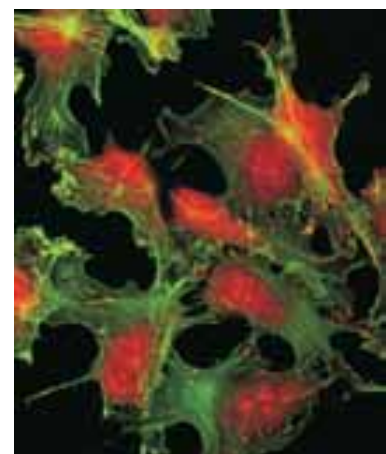
For the examination of tiny cells or proteins, a high-class microscope is an excellent tool. Common microscopes only provide a two-dimensional image of a cell compound, but biologists prefer a 3-D-view of cells and their interactivities. That's the specialty of a confocal fluorescence microscope (picture 1). Contrary to standard white-light microscopes, appearance of red-shifted fluorescence is examined and allocated to cellular components of interest. Obviously, spectral filters and wavelength-dependent optical devices are essential for such a set-up. Moreover, confocal fluorescence microscopes take advantage of a pinhole in the observation light path to suppress any fluorescence light from outside the focal plane. In order to achieve a 3-D image instead of a 2-D one, the complete optical assembly (focusing lens, dichroic mirror, pinhole and detector) can be moved in Z direction with highest accuracy. Merging a plurality of images from different focal depths, the biologist receives either a composite view or a 3-D view of his specimen with sub-micron resolution (picture 2). Furthermore, the 3-D image enables observation of the scanned object from a different position by rotating the image in the computer (x-z cross-sectional view).

Still there are two obstacles remaining:

- How to distinguish an object of interest (for instance protein) from an unimportant object?



Picture 1: Principle of a confocal microscope (courtesy from Zeiss)



Picture 2: image of fibroblasts

- How to focus sufficient light on the specimen for appropriate signal-to-noise ratio?

Laser-induced fluorescence

There are two approaches to generate fluorescent molecules in the cell:

- Either a combination of a human protein (e.g. GFAP) plus a fluorescent protein (e.g. CFP) is entered into the DNA of a cell, resulting in production of such fluorescent proteins (GFAP-CFP) by the cell itself. As such proteins stay in specific cell areas (e.g. the cytoskeleton, picture 3), the region of interest is clearly marked by the referring fluorescence.
- Or the fluorescent marker is injected into a cell compound and links to an object of interest, e.g. cell nucleus. Again, the referring fluorescence clearly indicates the position or travel of the tagged object.

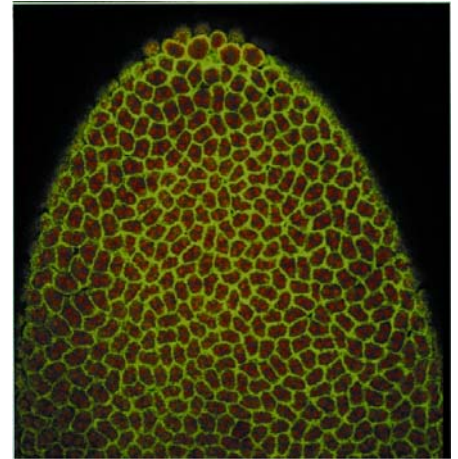
If such marker is excited by a light source which matches the marker's absorption band (e.g. 400 nm at Cascade blue), part of the fluorescence (which is always red-shifted, e.g. 420 nm at Cascade blue) reaches the detector. Any light from above or below the focal plane is blocked by the pinhole. In addition, the excitation light is deflected by the dichroic mirror and filters and thus separated from the fluorescence signal.

Which light source should be used for excitation? It needs to provide sufficient power to excite the fluorescent markers within short time. Furthermore, the beam quality should be excellent to focus all light onto a small spot in the (sub-)micron range. Finally, the wavelength needs to match the absorption bands of the dye. All such requirements can be met by sophisticated laser systems.

Techniques in fluorescence microscopy:

Several mechanisms and techniques are based on fluorescence microscopy:

- FLIM (fluorescence lifetime imaging microscopy) – can separate two dyes with similar fluorescence wavelength by additionally analyzing different fluorescence lifetime.
- FLIP (fluorescence loss in photo bleaching) – applies a strong laser pulse onto a spot of the specimen (to bleach the dye) and records the decrease of fluorescence at another spot to examine dye movement along the specimen. Transport processes in cells can be observed much easier this way.
- FRAP (fluorescence recovery after photo bleaching) applies a strong laser pulse onto a spot of the specimen (to bleach the dye) and records the increase of fluorescence at this spot to examine dye movement. Again, observation of transport processes is supported by this method, similar to FLIP.
- FRET (fluorescence resonance energy transfer) – detects the closeness of two different markers (and its labeled objects) if



Picture 3: Cytoskeleton of Drosophila embryo (courtesy from Zeiss)



Picture 4: Set-up of modern confocal laser-scanning microscope (courtesy from Zeiss)



Picture 5: Laser Scanning Microscope LSM 5 Live (courtesy from Zeiss)

the distance falls below a critical limit (approx. 10 nm). In such case fluorescence of the 2nd marker is detected. Using this method a biologist can observe approach of dyes (distance < 10 nm) far below the confocal resolution limit (200 nm) in living cells, for instance protein-protein interactions.

- STED (stimulated emission depletion) – surpasses the diffraction barrier of Abbe's law by applying two synchronized laser pulses onto a diffraction-limited spot on the specimen. The first laser pulse excites the dye, while the second laser pulse (red-shifted, doughnut-mode) depletes the fluorescence in the periphery of the spot. Only the fluorescence from the spot center is recorded by the microscope detector, leading to a previously unrivalled resolution (down to 20 nm was already demonstrated in the lab, compared to 200 nm for modern confocal microscopes).

Another well-known method in fluorescence microscopy is TIRF (total internal reflection fluorescence). However, TIRF is not part of confocal microscopy.

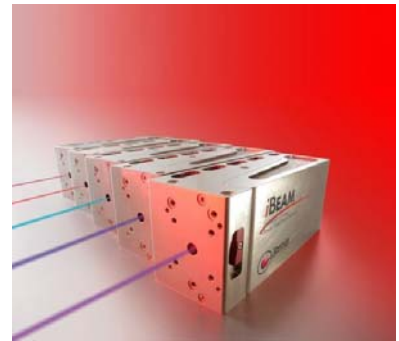
All these techniques are not part of this application report and will be subject to separate reports. The report in hand is related to standard confocal fluorescence microscopy.

What laser does a confocal microscope need?

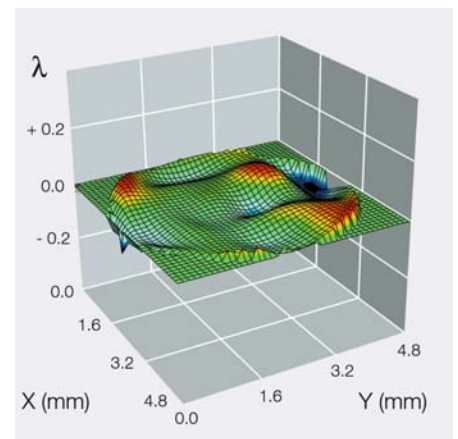
Confocal laser scanning microscopes (CLSM, picture 4 and 5) typically use attached laser boxes containing 3 to 6 laser systems. These laser lines are coupled into the same single-mode polarization-maintaining fiber. Obviously, this set-up prefers lasers which meet following requirements:

- High coupling efficiency, resulting from two properties:
 - Diffraction-limited beam quality. The better the beam quality, the better the focusability and subsequently the coupling efficiency.
 - Best beam pointing stability. Any change in pointing direction immediately causes reduced coupling efficiency.
- Highest output power. In order to speed up scanning, power is an important issue as the excitation energy per dye molecule stays constant. For bleaching applications such as FLIP or FRAP highest power is just good enough.
- Highest power stability. Any power drift during excitation results in an emission power drift and subsequently lower repeatability or minor reliability of the acquired data.
- Compact size. The smaller the size, the higher the number of integrated laser systems which enables more detailed examination of specimen.

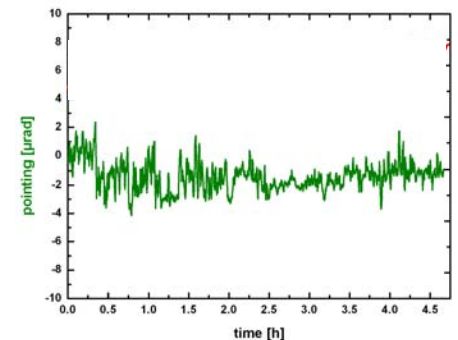
If still no space for extension of the laser box is available, additional fiber ports act as a versatile solution to cater for



Picture 6: iBeam series and its various wavelengths



Picture 7: Superb beam quality of iBeam 405 (wavefront error < 0.05 lambda)



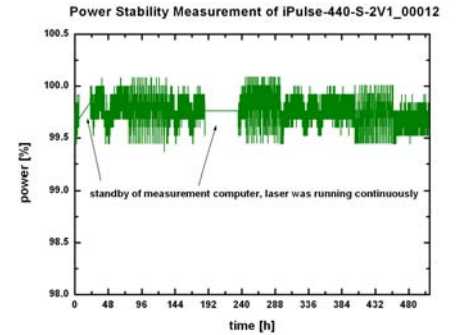
Picture 8: Beam pointing stability of iBeam series under constant ambient conditions

additional laser lines. Again, this emphasizes the importance of high fiber coupling efficiency.

Solution based on diode technology

TOPTICA gained vast experience in confocal microscopy which results in best-tailored diode lasers for this application. The iBeam series (picture 6) caters to the market demands by offering several key advantages:

- Superb beam quality to enable high fiber-coupling efficiency at single-mode fibers. Various diode laser manufacturers measure M^2 values to determine the beam quality. TOPTICA prefers wavefront measurements to separate aberration effects and thus achieve a top level of beam quality (wavefront error $< 0.05 \lambda$, picture 7)
- Excellent beam pointing stability of $< 10 \mu\text{rad/K}$ (picture 8) to avoid subsequent beam walk and thus reduced coupling efficiency. A beam walk of 1 micron can easily reduce the SM coupling efficiency by 10 to 30%.
- Highest power (e.g. 70 mW at 405 nm, 75 mW at 660 nm, 18 mW at 375 nm) with single diodes for fastest scanning. Even live videos can be recorded if the available power is sufficient.
- Unmatched power stability (power drift $< 0.5\%$ over 48 hours, picture 9) to achieve highest reproducibility of images and constant signal-to-noise ratio.



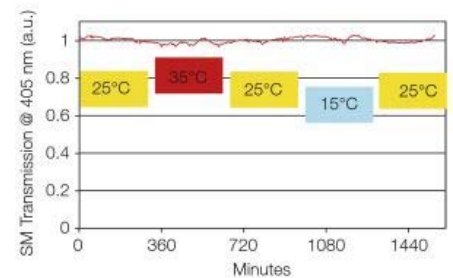
Picture 9: iBeam power stability (480 hours) under constant ambient conditions

Beyond these key advantages, our patented fiber-coupler FiberDock (picture 10) converts the standard free-beam module into a thermally insensitive fiber-coupled laser system. FiberDock cycling tests have proven a power drift of less than 2%, even when changing ambient temperatures by more than $10 \text{ }^\circ\text{C}$ (picture 11). Coupling efficiencies up to 87% were already demonstrated in the lab.



Picture 10: Versatile fiber coupler FiberDock

On the one hand, diode modules replace gas lasers due to their inherent features (lowest power consumption, no water cooling necessary, no vibration). Typical examples are 405 nm diode modules replacing 413 nm Krypton lasers or 635 nm diodes replacing 633 nm Helium-Neon lasers. On the other hand, many fluorescent dye producers also tailored specific markers especially for new diode wavelengths, e.g. Cascade blue, Alexa 405 or Pacific blue for 405 nm excitation.



Picture 11: Power stability at end of SM fiber

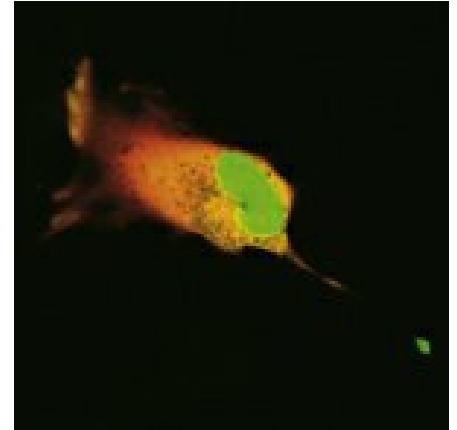
Trends into the future

Latest trends in confocal microscopy are photo-activation and photo-conversion. At photo-activation a violet laser pulse is applied to single PA-GFP dye molecules. This activation increases the fluorescence emission intensity by factor 100 when stimulated by a

blue laser. Now biologists can examine dynamic processes in a cell or along an organism in very detail by activating and selecting individual dyes.

At photo-conversion, again a violet laser pulse is applied to a single dye molecule (e.g. KAEDE, picture 12). As a consequence, the dye's fluorescence emission changes from green to red (by factor 2000) when irradiated by a blue laser. Contrary to photo-activation, structures marked by KAEDE already fluoresce before photo-conversion. Analysis of transport processes on cellular or subcellular level are thus much easier.

The iBeam is well prepared for the tasks of tomorrow. By providing up to 75 mW at 660 nm it presents itself as an ideal excitation source for many dyes (e.g. Cy 5, APC) previously stimulated only by red HeNe lasers. The iBeam wavelengths 405 nm and 440 nm address the dyes Cascade Blue, Pacific Blue, Alexa 405 and eCFP, Fura Red, respectively. On special request, the iBeam provides even longer wavelengths to support latest near-IR fluorescent markers.



Picture 12: KAEDE transfected cell, irradiated by 405 nm laser at region of interest (courtesy from Zeiss)