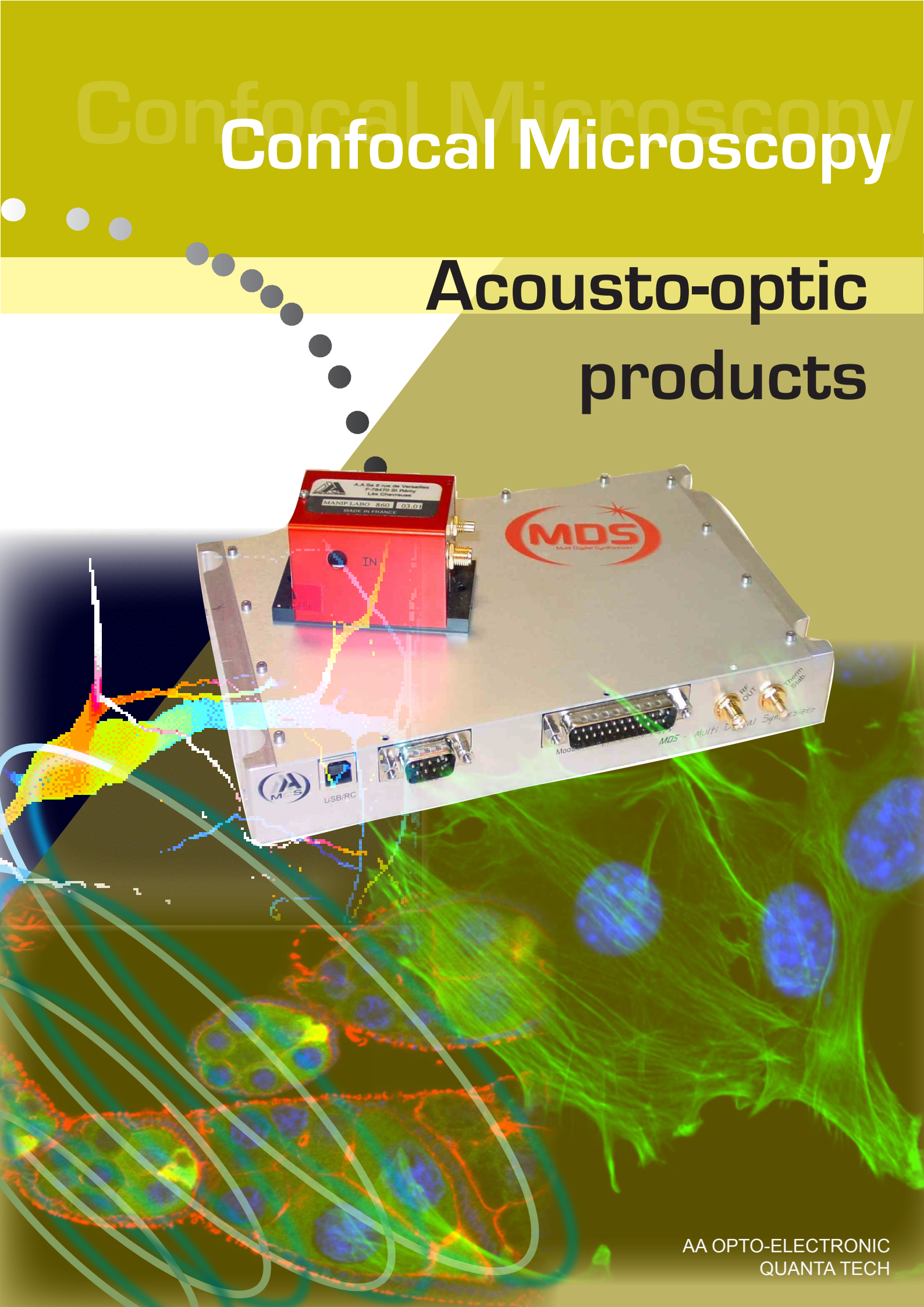
Confocal Microscopy

Acousto-optic products



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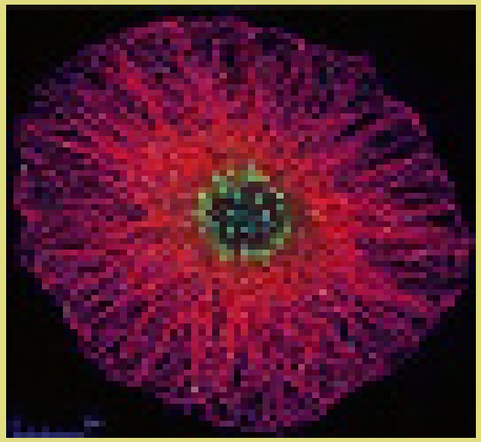
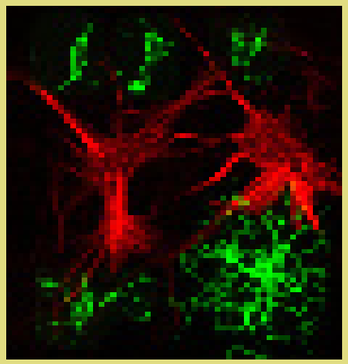
Introduction

Confocal microscopy is an imaging technique used to increase micrograph contrast and/or to reconstruct three-dimensional images by using a spatial pinhole to eliminate out-of-focus light or flare in specimens that are thicker than the focal plane. This technique has been gaining popularity in the scientific and industrial communities. Typical applications include life sciences and semiconductor inspection.



Basic concept

The principle of confocal imaging was patented by Marvin Minsky in 1961. In a conventional (i.e., wide-field) fluorescence microscope, the entire specimen is flooded in light from a light source. Due to the conservation of light intensity transportation, all parts of specimen throughout the optical path will be excited and the fluorescence detected by a photodetector or a camera. In contrast, a confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus information. Only the light within the focal plane can be detected, so the image quality is much better than that of wide-field images. As only one point is illuminated at a time in confocal microscopy, 2D or 3D imaging requires scanning over a regular raster (i.e. a rectangular pattern of parallel scanning lines) in the specimen. The thickness of the focal plane is defined mostly by the square of the numerical aperture of the objective lens, and also by the optical properties of the specimen and the ambient index of refraction.



Different principles

Three types of confocal microscopes are commercially available: Confocal laser scanning microscopes, spinning-disk (Nipkow disk) confocal microscopes and Programmable Array Microscopes (PAM). Generally speaking, confocal laser scanning microscopy yields better image quality but the imaging frame rate is very slow (less than 3 frames/second); spinning-disk confocal microscopes can achieve video rate imaging—desired for dynamic observations.

Confocal Laser scanning Microscopy

Confocal laser scanning microscopy (CLSM or LSCM) is a valuable tool for obtaining high resolution images and 3-D reconstructions. The key feature of confocal microscopy is its ability to produce blur-free images of thick specimens at various depths. Images are taken point-by-point and reconstructed with a computer, rather than projected through an eyepiece. The principle for this special kind of microscopy was developed by Marvin Minsky in 1953, but it took another thirty years and the development of lasers for confocal microscopy to become a standard technique toward the end of the 1980s.

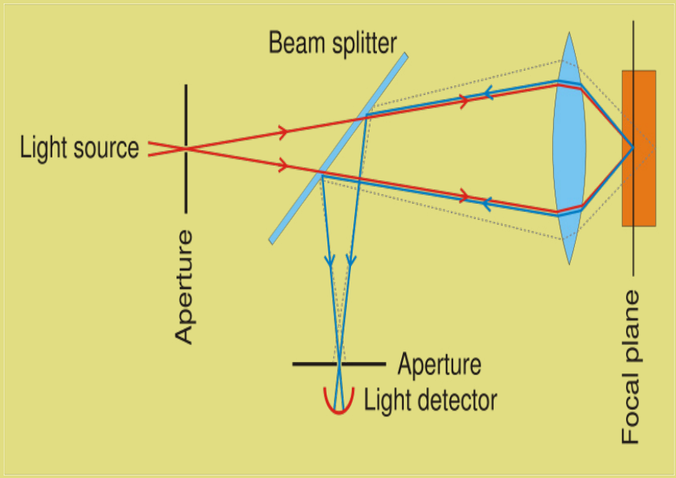


Image formation

In a laser scanning confocal microscope a laser beam passes a light source aperture and then is focused by an objective lens into a small (ideally diffraction-limited) focal volume within a fluorescent specimen. A mixture of emitted fluorescent light as well as reflected laser light from the illuminated spot is then recollected by the objective lens. A beam splitter separates the light mixture by allowing only the laser light to pass through and reflecting the fluorescent light into the detection apparatus. After passing a pinhole the fluorescent light is detected by a photo-detection device (photomultiplier tube (PMT) or avalanche photodiode) transforming the light signal into an electrical one which is recorded by a computer.

The detector aperture obstructs the light that is not coming from the focal point, as shown by the dotted grey line in the image. The out-of-focus points are thus suppressed: most of their returning light is blocked by the pinhole. This results in sharper images compared to conventional fluoresence microscopy techniques and permits one to obtain images of various z axis planes (z-stacks) of the sample.

The detected light originating from an illuminated volume element within the specimen represents one pixel in the resulting image. As the laser scans over the plane of interest a whole image is obtained pixel by pixel and line by line, while the brightness of a resulting image pixel corresponds to the relative intensity of detected fluorescent light. The beam is scanned across the sample in the horizontal plane using one or more (servo-controlled) oscillating mirrors. This scanning method usually has a low reaction latency and the scan speed can be varied. Slower scans provide a better signal to noise ratio resulting in better contrast and higher resolution. Information can be collected from different focal planes by raising or lowering the microscope stage. The computer can generate a three-dimensional picture of a specimen by assembling a stack of these two-dimensional images from successive focal planes.

In addition, confocal microscopy provides a significant improvement in lateral resolution and the capacity for direct, non-invasive serial optical sectioning of intact, thick living specimens with an absolute minimum of sample preparation. As laser scanning confocal microscopy depends on fluorescence, a sample usually needs to be treated with fluorescent dyes to make things visible. However, the actual dye concentration can be very low so that the disturbance of biological systems is kept to a minimum. Some instruments are capable of tracking single fluorescent molecules. Additionally transgenic techniques can create organisms which produce their own fluorescent chimeric molecules. (such as a fusion of GFP, Green fluorescent protein with the protein of interest).

Resolution enhancement by the confocal principle

Laser scanning confocal microscopy (LSCM) is a scanning imaging technique in which the resolution obtained is best explained by comparing it with another scanning technique like Scanning electron microscope (SEM). Not to be confused with phonograph-like imaging—AFM or STM, for example, where the image is obtained by scanning with an atomic tip over a conducting surface.

In LSCM a fluorescent specimen is illuminated by a point laser source, and each volume element is associated with a discrete fluorescence intensity. Here, the size of the scanning volume is determined by the spot size (close to diffraction limit) of the optical system. This is due to the fact that the image of the scanning laser is not an infinitely small point but a three-dimensional diffraction pattern.

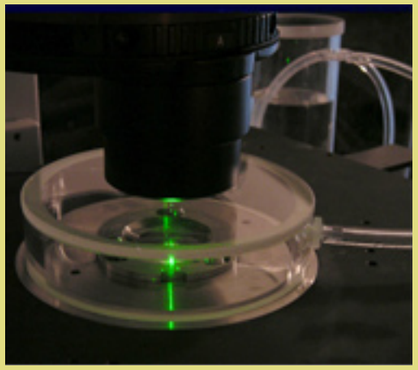
The size of this diffraction pattern and the focal volume it defines is controlled by the numerical aperture of the system's objective lens and the wavelength of the laser used. This can be seen as the classical resolution limit of conventional optical microscopes using wide-field illumination. However, with confocal microscopy it is even possible to overcome this resolution limit of wide-field illuminating techniques as only light generated in a small volume element is detected at a time. Here it is very important to note that the effective volume of light generation is usually smaller than the volume of illumination; that is, the diffraction pattern of detectable light creation is sharper and smaller than the diffraction pattern of illumination.
In other words, the resolution limit in confocal microscopy depends not only on the probability of illumination but also on the probability of creating enough detectable photons, so that the actual addressable volume being associated with a generated light intensity is smaller than the illuminated volume. Depending on the fluorescence properties of the used dyes, there is a more or less subtle improvement in lateral resolution compared to conventional microscopes. However, by using light creation processes with much lower probabilities of occurrence such as second harmonic generation (SHG), the volume of addressing is reduced to a small region of highest laser illumination intensity resulting in a significant improvement in lateral resolution.

Unfortunately, the probability decrease in creation of detectable photons has a bad effect on the signal to noise ratio. This can be compensated by using more sensitive photo-detectors or by increasing the intensity of the illuminating laser point source. Increasing the intensity of illumination latter risks excessive bleaching or other damage to the specimen of interest, especially for experiments in which comparison of fluorescence brightness is required.

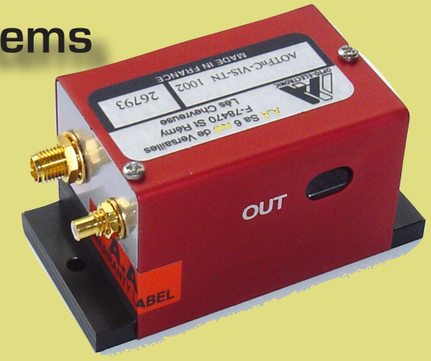
Uses

Confocal microscopy is clinically used in the evaluation of various eye diseases. It is particularly useful for imaging, qualitative analysis and quantitafication of endothelial cells of the cornea. It is used for localising and identifying presence of filamentary fungal elements in the corneal stroma in cases of keratomycosis, enabling rapid diagnosis and thereby early institution of definitive therapy.

Confocal microscopy is also used as the data retrieval mechanism in some 3D optical data storage systems and has helped determine the age of the Magdalen papyrus



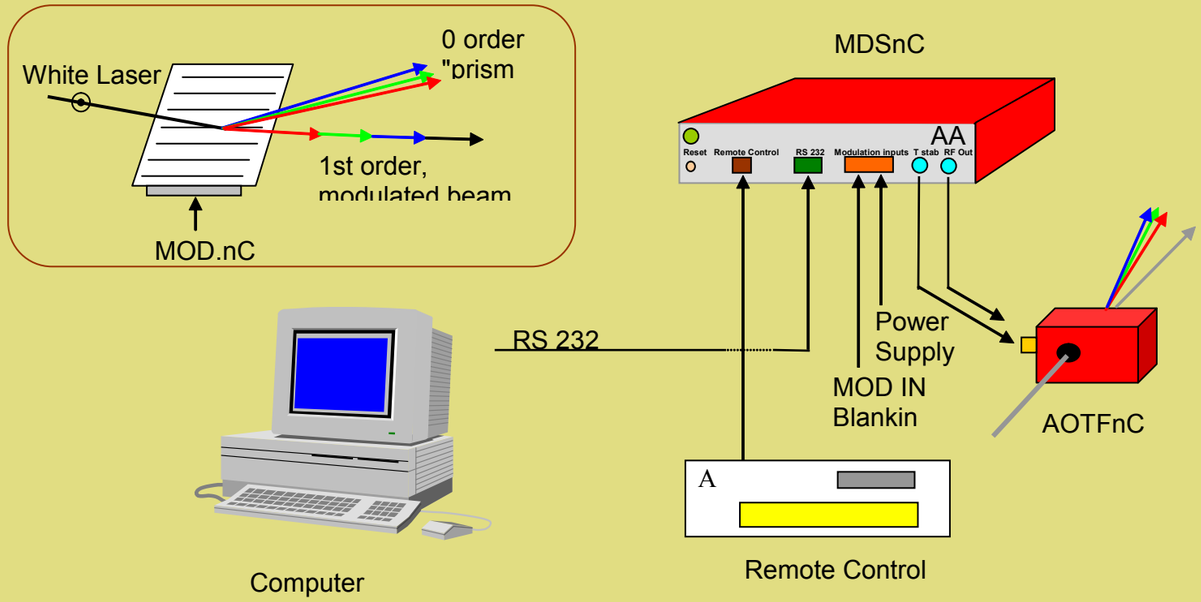
Polychromatic Modulation Systems



The AOTF.nC is a special acousto-optic tunable filter which uses the anisotropic interaction inside a tellurium dioxide crystal to control independently or simultaneously different lines from an incoming laser light (White laser, Ar+, Kr+, HeNe, DPSS, Dye...).
Up to 12 distinct lines can be mixed and separately modulated in order to generate different colorimetric patterns.

The specific crystal cut of the AOTF.nC produces good diffraction efficiency (> 90%), narrow resolution (1-2 nm), a low cross-talk between lines, and high extinction ratio.
The large separation angle between 0 and 1st orders, as well as the excellent output chromatic colinearity (<0.2 mrd over 450-700 nm) make this AOTF a powerful tool for free space or fiber pigtailed applications.
Its associated thermal stabilisation maintains stable diffraction efficiency and reduces dramatically beam drift with single mode fiber pigtailing. This is a major advantage for high sensitivity applications.

The associated driver MOD.nC, based on PLLs (Phase Locked Loop), has been specially designed in order to exploit the best of the AOTF.nC features.
Its compact design with single power supply, low RF emissions and ease of use will satisfy the most demanding of applications, where accuracy and flexibility are key requirements.
Thanks to its complete numerical design and integrated microcontroller setting up is fast, simple and repeatable.
Access to and adjustments of functions is simple with either a bright LCD display (with remote control adjustment) or through a RS232 serial link (with computer control).
All parameters are stored in an EEPROM and are automatically loaded after each switch on.
Each line is externally controlled by a distinct modulation input signal which can be TTL or analog. Additionally, all lines can be simultaneously controlled by a blanking signal which produces smooth effects without modifying the colorimetric balance.
The combination of the modulation input and blanking signals provides the best extinction ratio performance (> 140 dB).

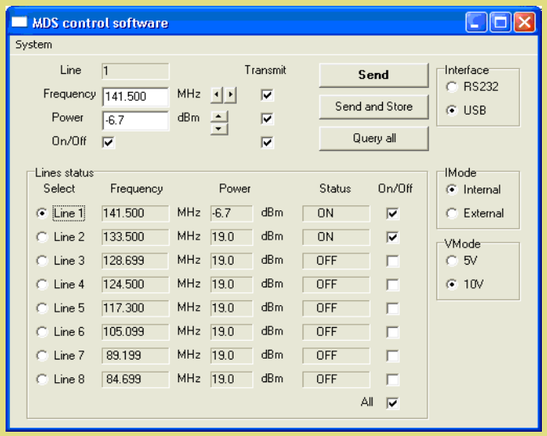


Polychromatic Modulators

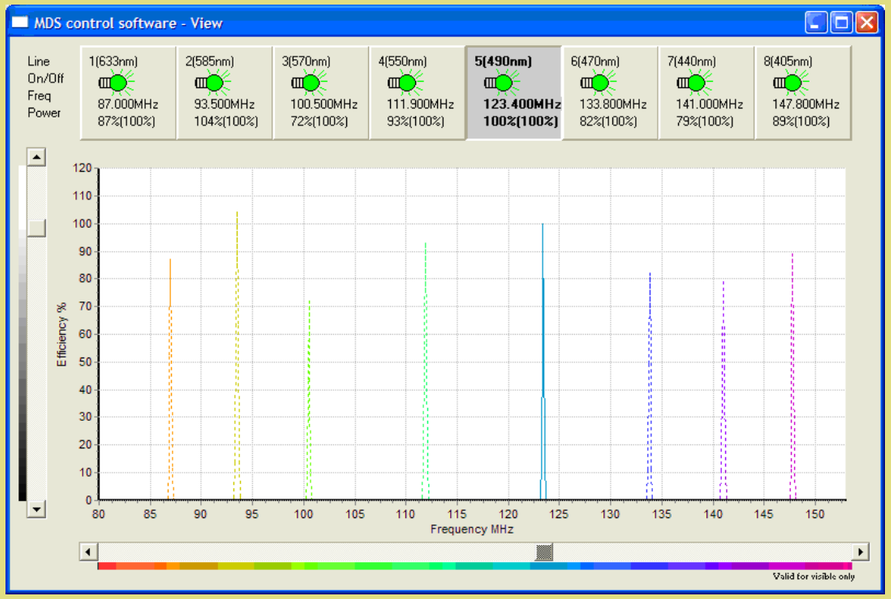
AOTFnC	UV	VIS	VIS
Number of channels / Lines	4	8	8
Acoustic velocity (nom)	675 m/s	650 m/s	660 m/s
Optical wavelength range	350-430 nm	450-700 nm	400-650 nm
Transmission	> 80 % -nom 90%	> 95 %	> 90 %
AO interaction type	Birefringent	Birefringent	Birefringent
Selected order	+1	-1	-1
Input Light polarization	Linear parallel	Linear orthogonal	Linear orthogonal
Output Light polarization	Linear orthogonal	Linear parallel	Linear parallel
Drive frequency range	110-180 MHz	80-153 MHz	74-158 MHz
Active aperture	2 x 2 mm²	3 x 3 mm²	3 x 3 mm²
Spectral resolution (FWHM)		nom 1-2 nm	nom 1-4 nm
Separation angle (orders 0-1)	> 4.2 degrees	> 4.6 degrees	> 4 degrees
Chromatic colinearity (order 1)	< 0.2 mrd @351+363 nm	< 0.2 mrd	< 0.3 mrd
Temperature stabilization	T or TN	T or TN	T or TN
AO Efficiency	>=90%	>= 90 % /line	>= 90 % /line
Rise time	980 ns / mm	1010 ns / mm	1000 ns /mm
Max accepted RF power	< 1 W all lines	< 1 W all lines	nom 1 W all lines
Electrical impedance	50 Ohms	50 ohms	50 ohms
VSWR	< 2/1	< 2/1	< 2/1
Size		70 x 36.6 x 35.8 mm3	
Operating temperature		10 to 40 °C	

Polychromatic Drivers / Digital versions

These drivers based on Direct Digital Synthesizers (DDS), produce multiple fixed stable and accurate RF frequency signals for polychromatic modulators. Their brand new design with “on the edge” technology offers unique performance in term of accuracy, speed and stability (single/multi-line), thanks to their internal temperature correction and high linearity design.
The built in amplifier delivers the necessary RF power to drive the acousto-optic device, with reduced power consumption (AA “COLD DESIGN”).
The RF output power per channel can be individually modulated (MOD IN signals) or simultaneously modulated (BLANKING signal). AA focussed on a ultra low crosstalk version with superior fast and fall time.
The adjustments of the driver (Frequency & Power) can be done with a remote control, USB or through RS 232 communication to allow user flexibility in power control or frequency scanning.



USB Software



MDSnC

Number of channels	Up to 8
Frequency range	Octave or above in 20-180MHz – will be adapted to AO
Frequency stability	+/- 2 ppm/°C
Frequency accuracy	Nom 1 KHz
Frequency step	Nom 1 KHz
Frequency control	Remote Control or USB, Option : RS232
Power Supply	OEM version : 24 VDC - nom 0.85A
Laboratory version	110/230 VAC - 50-60 Hz
Rise Time / Fall time (10-90 %)	< 50ns
Modulation Input Control	Analog 0-5 V or 0-10 V / 10 KOhms
Blanking Input Control	Analog 0-5 V or 0-10 V / 10 KOhms
Extinction ratio @ 125 MHz	MOD IN > 80 dB typ 90dB
	BLK > 70 dB typ 80dB
	MOD IN + BLK > 90 dB typ 100 dB
Output RF power	22 dBm per channel
Output Impedance	50 Ohms
V.S.W.R.	Nom < 1.5/1
Input / Output connectors	DB25 / SMA (DB9 for RS232)
Size	OEM version : 207 x 127 x 20.2 mm3
Laboratory version	Rack 19", 1U
Weight	OEM version : nom 1 kg
Laboratory version	nom 4 kg
Heat exchange	OEM version : Conduction
Laboratory version	stand alone
Operating temperature	10 to 40 °C
Maximum case temperature	OEM version : 50 °C
Option	Cover with Fan