For the signal-idler excitation scheme the suitable dichroic mirrors chosen that allow covering the most part of the spectral region between about 700 cm^{-1} and 3400 cm^{-1} are:

- The dichroic mirror 970DCSXPR by Omega Optical, for simplicity named DM970 since the cut-off wavelength is placed at about 970 nm, allows a CARS imaging in the spectral region between about 700 and 1200 cm^{-1}.
- The bandpass filter ET890/220M by Chroma Technologies tilted by 45°, for simplicity named P890-220-45°, allows a CARS imaging in the spectral region between about 1250 and 2700 cm^{-1}.
- The shortpass filter E950SP-2P by Chroma Technologies tilted by 45°, for simplicity named SP950-45°, allows a CARS imaging in the spectral region between about 1800 and over 3400 cm^{-1}.

It should be noted that there are two uncovered regions (green rectangles in Fig. 3.35), one for wavenumber lower than about 700cm^{-1} and another between 1200 and 1250 cm^{-1}, this because I considered to be acceptable a reflectivity higher than 80% (transmittance lower than 20%) for a good excitation of the sample and a transmittance higher than 70% (reflectivity lower than 30%) for a good detection of the CARS/TPEF/SHG signal. If it is considered a reflectivity higher than about 75% (transmittance lower than about 25%) using the dichroic mirror DM970 it will be possible to extend the CARS imaging in the spectral region between about 700 and 1300 cm^{-1}, covering also the region between 1200 and 1250 cm^{-1}. Thus it could be concluded that using these three dichroic mirrors it is possible to cover the spectral region between about 700 and 3400 cm^{-1}.

Wavenumber [cm^{-1}]	Idler [nm]	Signal [nm]	CARS [nm]
1323.7	1144.6	994	878.4
1303.5	1143.3	995	880.8
1283.3	1142.0	996	883.1
1263.2	1140.7	997	885.5
1243.1	1139.3	998	887.9
1223.0	1138.0	999	890.2
1203.0	1136.8	1000	892.6
1183.0	1135.5	1001	895.0
1163.1	1134.2	1002	897.4
1143.2	1132.9	1003	899.8
1123.3	1131.6	1004	902.2

Table 3.3 List of the CARS, signal and idler wavelengths in function of the CARS wavenumber in the region between about 1120 cm^{-1} and 1320 cm^{-1}.

For such regards the epi-detection of signals generated with other non-linear process such as TPEF and SHG, for each dichroic mirror there are some limitations because of the characteristic of their transmittances as reported in Table 3.4.

Dichroic mirror type	Epi-detection spectral region [nm]	CARS spectral Region [cm^{-1}]
DM970	<440-525 631-962	700-1300
P890-220-45°	732-884	1250-2700
SP950-45°	467-502 562-630 663-826	1800-3400

Table 3.4 Epi-detection spectral windows for the selected dichroic mirrors.

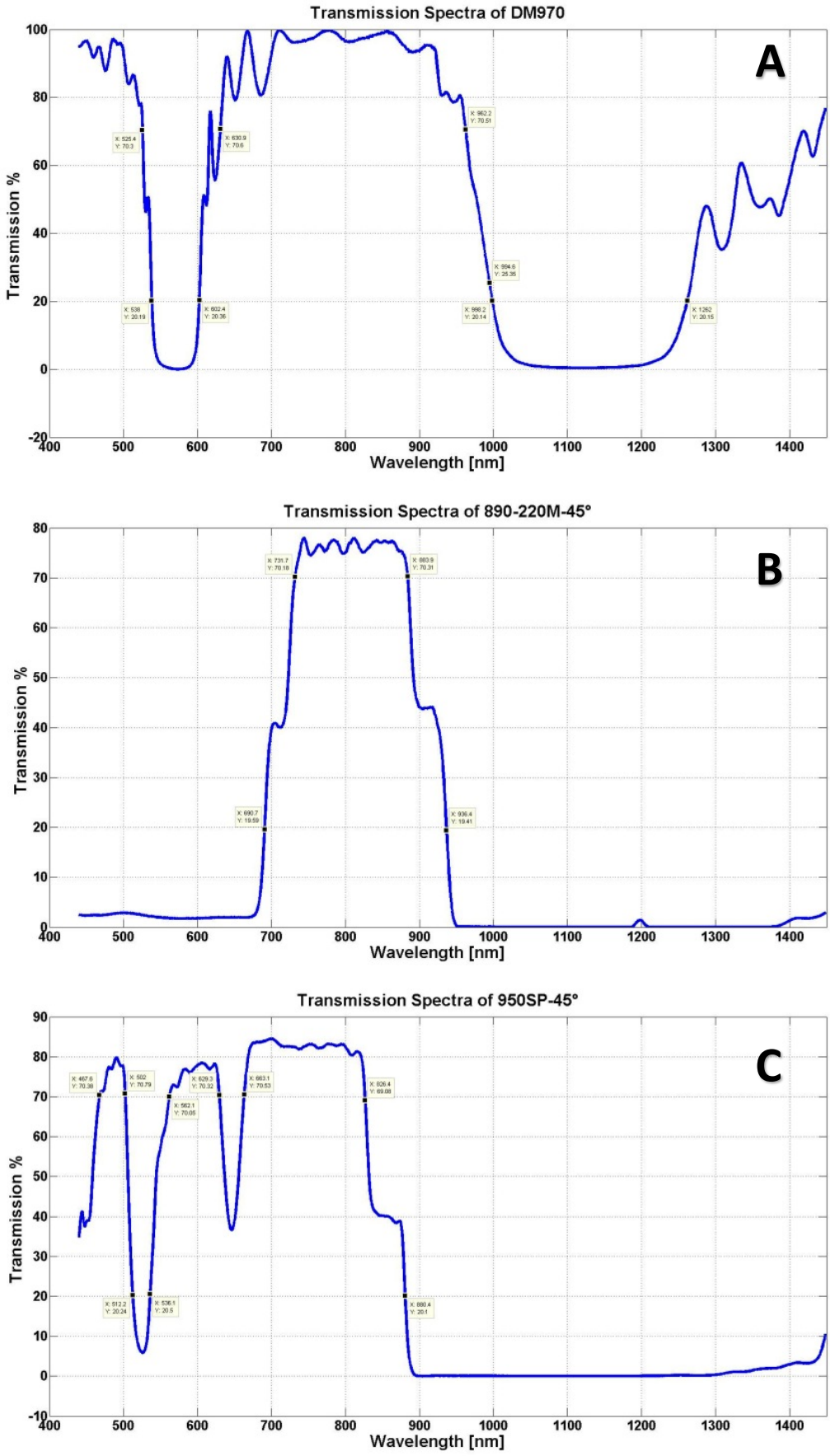

Fig. 3.37 Graphical transmission spectra of dichroic mirror DM970 (A), 890-220M-45° (B), 950SP-45° (C).

Using the signal-1064 excitation scheme have been chosen several dichroic mirrors that can be utilized to obtain also epi-detection CARS imaging, covering mostly a spectral region between about 950 and 3400 cm^{-1}. The dichroic mirrors selected are:

- The dichroic mirror DM970, allows a CARS imaging in the spectral region between about 500 and 600 cm^{-1}.
- The bandpass filter P890-220-45° tilted by 45°, allows a CARS imaging in the spectral region between about 950 and 1300 cm^{-1}.
- The shortpass SP950-45° tilted by 45°, allows a CARS imaging in the spectral region between about 1350 and over 1950 cm^{-1}.
- The dichroic mirror 850DCSXPR by Omega Optical, for simplicity named DM850 since the cut-off wavelength is placed at about 850 nm, allows a CARS imaging in the spectral region between about 1370 and 2100 cm^{-1}.
- The dichroic mirror DM740 was the one selected when the Olympus microscope BX51WI with the scan unit FV300 have been ordered, and it allows a CARS imaging in the spectral region between about 2150 and over 3400 cm^{-1}, ideal to analyse samples in the "CH region".

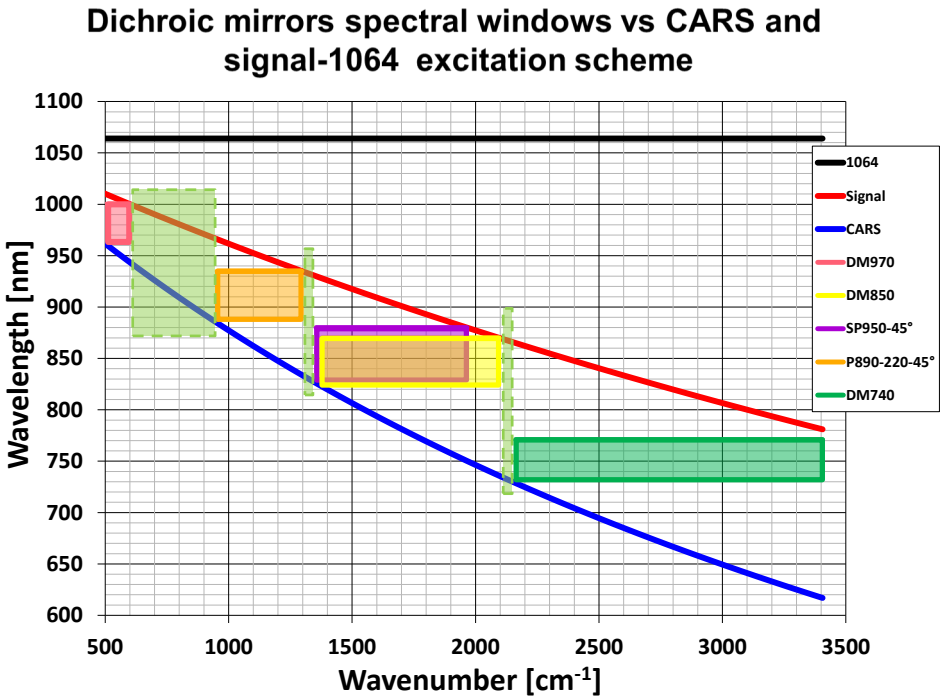

Fig. 3.38 Dichroic mirrors spectral windows versus CARS and signal-1064 excitation scheme.

With these dichroic mirrors it is not possible to cover the whole CARS spectrum and some regions of the CARS spectrum cannot be measured in epi-detection. These regions are:

- In the range between about 600 and 950 cm^{-1} (a dichroic mirror with a cut-off wavelength of about 950 nm which transmits shorter wavelengths and reflects longer wavelengths could solve this issue).
- In the range between 1300 and 1350 cm^{-1} (the dichroic mirrors P890-220-45° and SP950-45° could be used to cover this region but with a lower transmittance or a lower reflectivity).

- In the range between 2100 and 2150 cm^{-1} (the dichroic mirrors DM850 and DM740 could be used to cover this region but with a lower transmittance or a lower reflectivity).

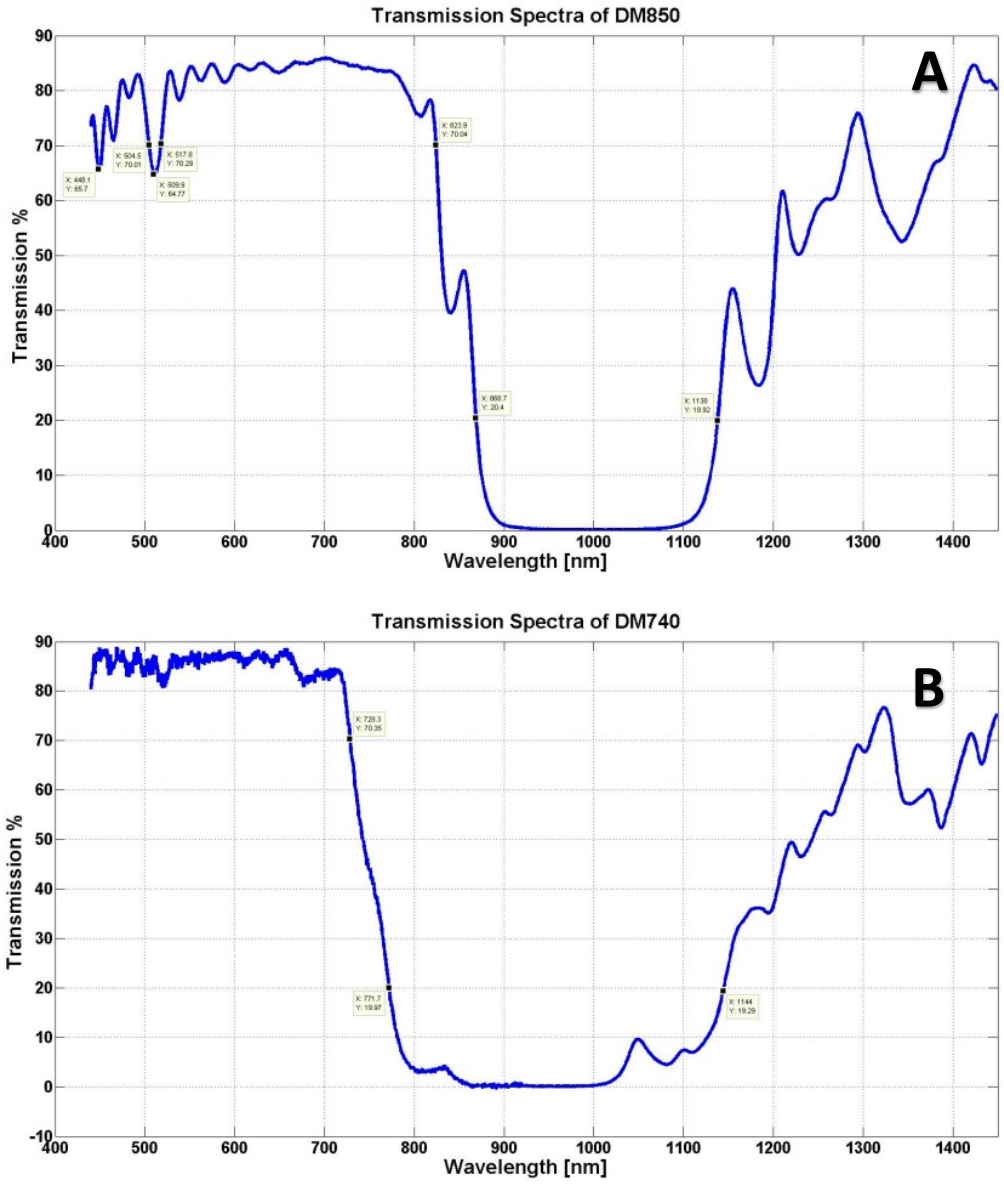

Fig. 3.39 Graphical transmission spectra of dichroic mirror DM850 (A), DM740 (B).

For such regards the epi-detection of signals generated with other non-linear process such as TPEF and SHG, for each dichroic mirror there are some limitations because of the characteristic of their transmittances as reported in Table 3.5.

Dichroic mirror type	Epi-detection spectral region [nm]	CARS spectral Region [cm^{-1}]
DM970	<440-525 631-962	500-600
P890-220-45°	732-884	950-1300
SP950-45°	467-502 562-630 663-826	1350-1950
DM850	<440-824	1370-2100
DM740	<440-728	2150-3400

Table 3.5 Epi-detection spectral windows for the selected dichroic mirrors.

The filter set-up of the scan head was chosen when the Olympus microscope system was ordered in a way to perform also imaging of samples stained with fluorescence dyes.

The scan head is equipped with two emission dichroic mirrors the DM570 and the DM630, which can be set exclusively.

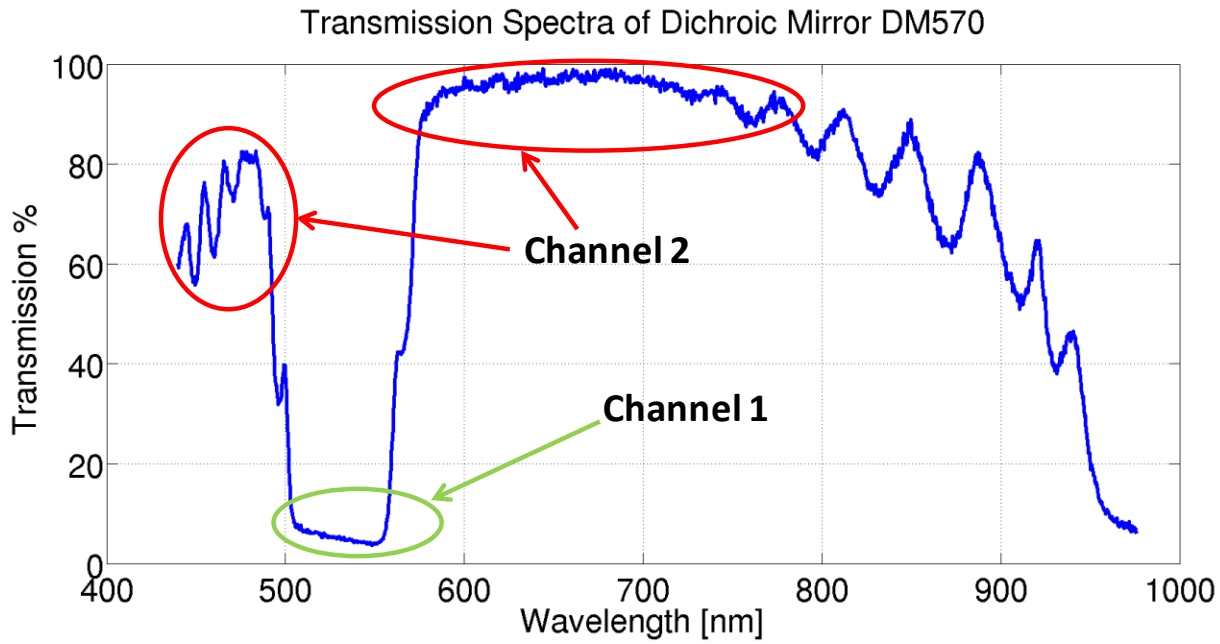

Fig. 3.40 Transmission spectrum of the scan head internal emission dichroic mirror DM570.

The dichroic mirror DM570 reflects to the first channel branch the fluorescence signal spectrum between 500-570 nm, while transmits to the second channel branch two spectral regions: between 430 nm (the lower limit of the spectrometer used) and 490 nm and between 570-740 nm.

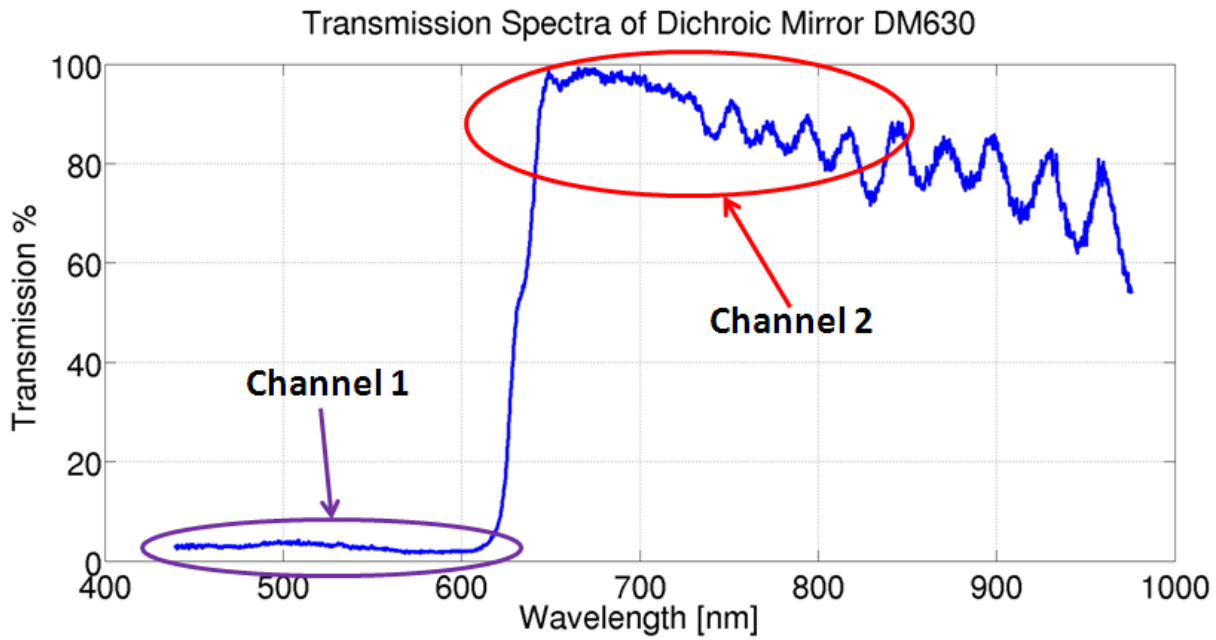

Fig. 3.41 Transmission spectrum of the scan head internal emission dichroic mirror DM630.

The dichroic mirror DM630 reflects to the first channel branch the fluorescence signal spectrum between 430-630 nm, while transmits to the second channel branch the spectral region between 630-740 nm. In each channel branch other two optical filters could be inserted in order to increase unwanted spectra rejection and improve the quality of the detected signal.

In the first channel the filters are:

- A short pass filter SP625 with a cut-off wavelength of about 625 nm;
- A barrier filter BF510IF which transmits wavelengths larger than 510 nm, cutting the blue region.

In the second channel the filters are:

- A short pass filter SP625 with a cut-off wavelength of about 625 nm;
- A barrier filter BA530RIF which transmits wavelengths below 530 nm.

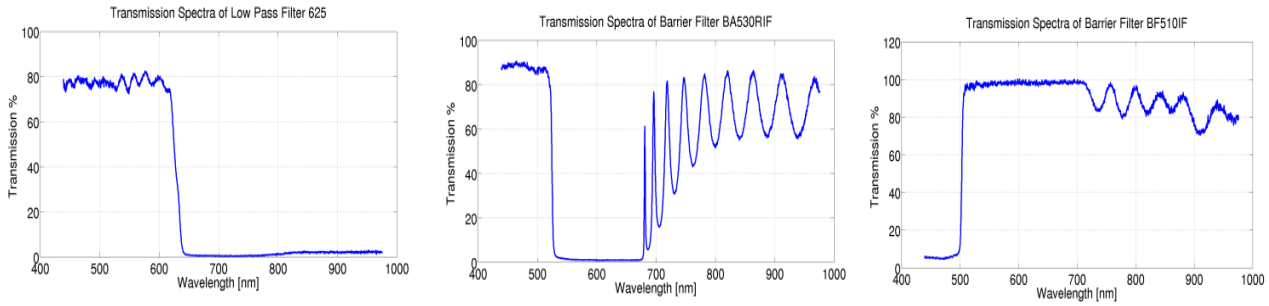

Fig. 3.42 Transmission spectra of the optical filters used in the two detection channels.

Moreover additional short pass filters can be placed before the confocal aperture in the scan head increasing the rejection of the excitation source.

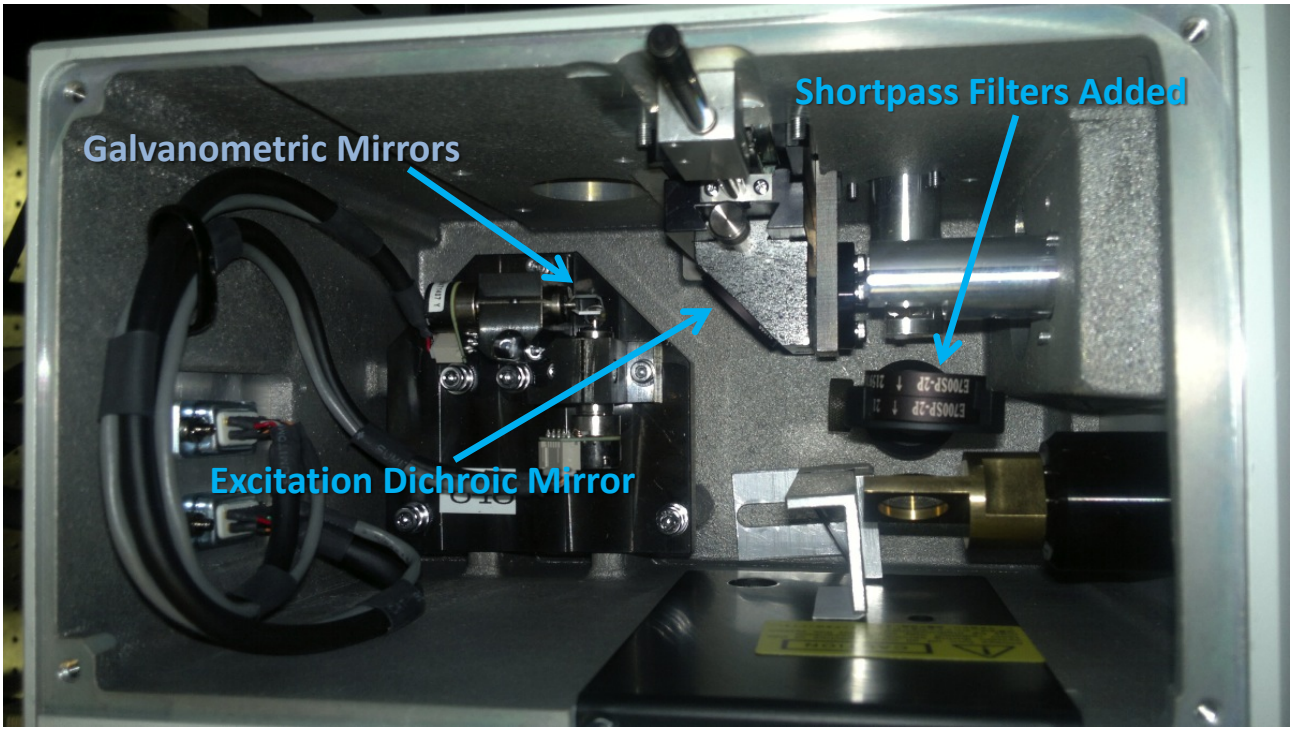

Fig. 3.43 Shortpass filters added in the microscope scan head.

Channel 1					
	Filter inserted			Transmitted Range [nm]	Note
DM570	SP625			490-570	Blue-Green
		BF510IF		*510-570**	Excitation source is not completely rejected
	SP625	BF510IF		510-570	Green
			SP700	490-570	Blue-Green
	SP625		SP700	490-570	Blue-Green
		BF510IF	SP700	510-570	Green
	SP625	BF510IF	SP700	510-570	Green
DM630	SP625			430-625	Blue-Green-Yellow-Orange
		BF510IF		*510-630**	Excitation source is not completely rejected
	SP625	BF510IF		510-630	Green-Yellow-Orange
			SP700	430-630	Blue-Green-Yellow-Orange
	SP625		SP700	430-630	Blue-Green-Yellow-Orange
		BF510IF	SP700	510-630	Green-Yellow-Orange
	SP625	BF510IF	SP700	510-630	Green-Yellow-Orange

Table 3.6 Spectral detectable ranges for channel one using all the possible filters combinations.

Channel 2					
	Filter inserted			Transmitted Range [nm]	Note
DM570	SP625			<430-490 570-625	Blue-Yellow-Orange
		BA530RIF		<430-490*	Excitation source is not completely rejected
	SP625	BA530RIF		<430-490	Blue
			SP700	<430-490 570-700	Blue-Yellow-Orange-Red
	SP625		SP700	<430-490 570-625	Blue-Yellow-Orange
		BA530RIF	SP700	<430-490	Blue
	SP625	BA530RIF	SP700	<430-490	Blue
DM630	SP625			-	
		BA530RIF		-	
	SP625	BA530RIF		-	
			SP700	630-700	Red
	SP625		SP700	630-700	Red
		BA530RIF	SP700	630-700	Red
	SP625	BA530RIF	SP700	-	

Table 3.7 Spectral detectable ranges for channel two using all the possible filters combinations.

Second harmonic generation filter set-up

The optical filters for SHG microscopy technique have been chosen considering the multimodal utilization of the microscope that integrates CARS and TPEF techniques too. In many biological applications that make use of CARS microscopy, cells are imaged using the resonant signal from CH_2 stretch vibration in lipids present in the cellular membrane and in the liposomes. The peak for the symmetric CH_2 stretch vibration appears at about 2840 cm^{-1} and it can be imaged tuning the OPO at about 924 nm for the signal and 1252 nm for the idler using the signal-idler excitation scheme, or at about 817 nm for the signal when it is used the signal-1064 excitation scheme. The SHG process has an increased efficiency with shorter wavelengths [67] thus it was chosen to use the OPO signal output and in certain particular applications the 1064 nm laser output as SHG excitation sources. At these conditions the resulting SHG signals generated are spectrally peaked at about 462 nm, 408.5 nm and 532 nm.

At this purpose three bandpass optical filters have been chosen:

- The model D455-70x (Chrome Technology Corp.) with transmission between 427 nm and 490 nm, this transmission range corresponds also to an excited CARS spectral region between about 1700 cm^{-1} and 4620 cm^{-1} using signal-idler excitation scheme and a range between 805 cm^{-1} and 2310 cm^{-1} using signal-1064 excitation scheme.
- The model ET405-20x (Chrome Technology Corp.) with transmission between 396 nm and 416 nm, this transmission range corresponds also to an excited CARS spectral region between about 2620 cm^{-1} and 3230 cm^{-1} using signal-1064 excitation scheme.
- The model FF01-531-22 (Semrock) with transmission between 518 nm and 542 nm that allow covering all the CARS spectral region using signal-1064 excitation scheme.

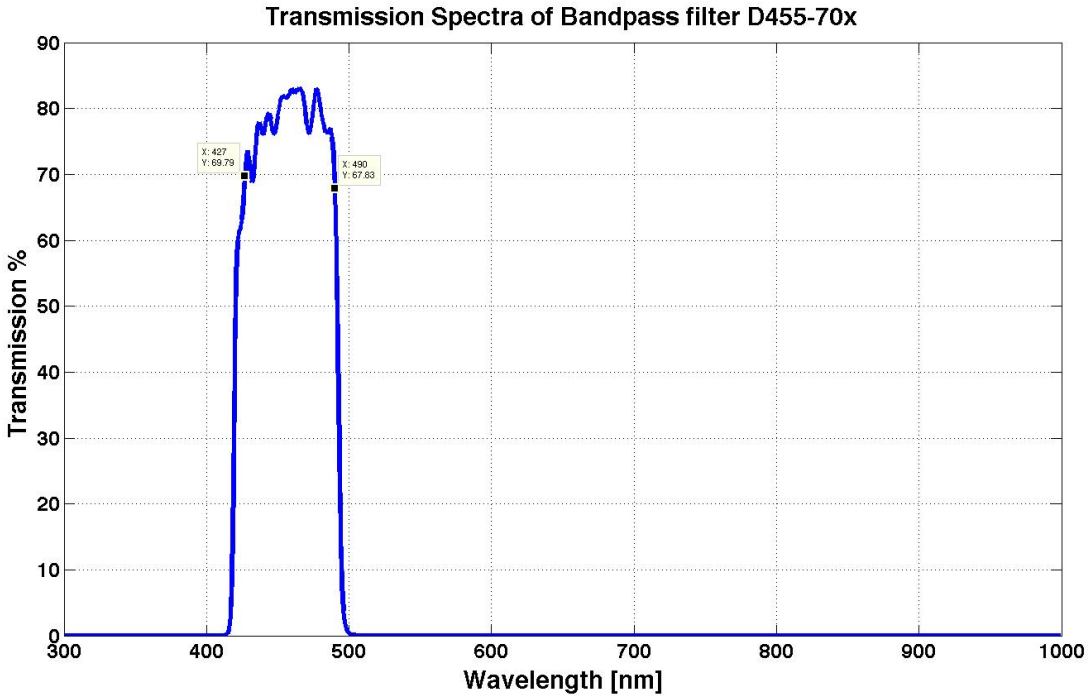

Fig. 3.44 Transmission spectra of the bandpass filter D455-70x.

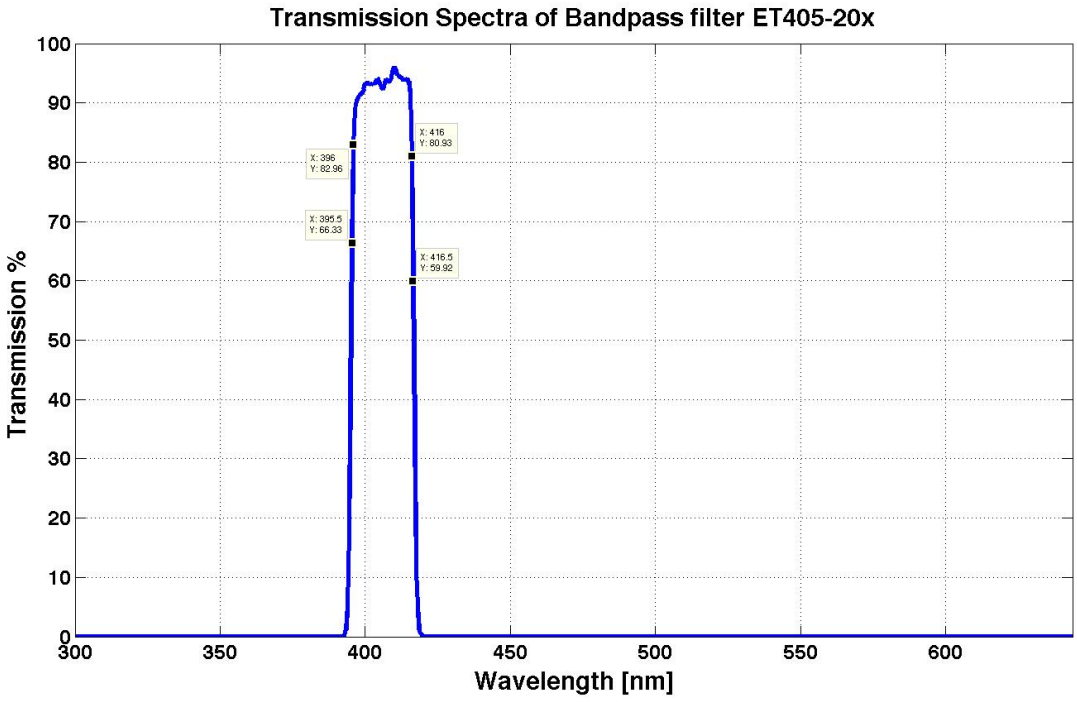

Fig. 3.45 Transmission spectra of the bandpass filter ET405-20x.

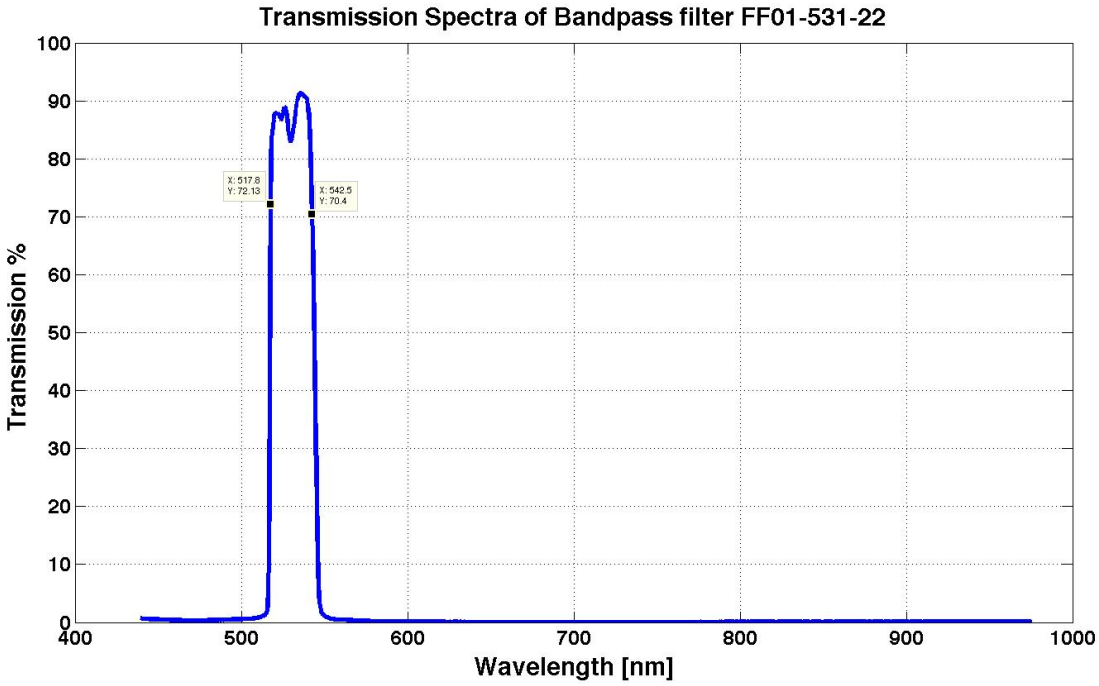

Fig. 3.46 Transmission spectra of the bandpass filter FF01-531-22.

In some applications it is convenient to use an excitation wavelength longer than for example 924 nm (that used for the signal to excite CH_2 stretch) in order to decrease the probability to have autofluorescence signal from the sample. For this reason also a bandpass filter with transmission between about 465 nm and 495 nm has been chosen, in a way to use an excitation signal with a wavelength in the range of 930 nm and 990 nm. That transmission range corresponds also to an excited CARS spectral region between about 1400 cm^{-1} and 2700 cm^{-1} using signal-idler excitation scheme and a range between 700 cm^{-1} and 1360 cm^{-1} using signal-1064 excitation scheme.

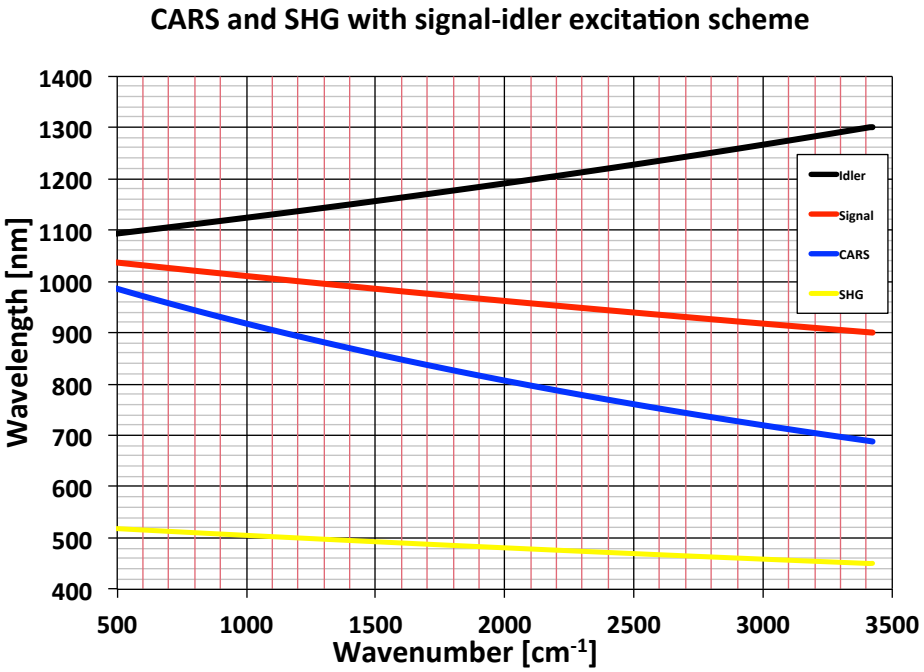

Fig. 3.47 Spectral characteristic of CARS and SHG signals with signal-idler scheme.

For the detection of the SHG signal the PMT is the best solution because as it can be observed from Fig. 3.30, the spectral sensitivity is higher in the region between 400 nm and 500 nm and the cut-off wavelength is at about 200 nm, quite far away the region of use.

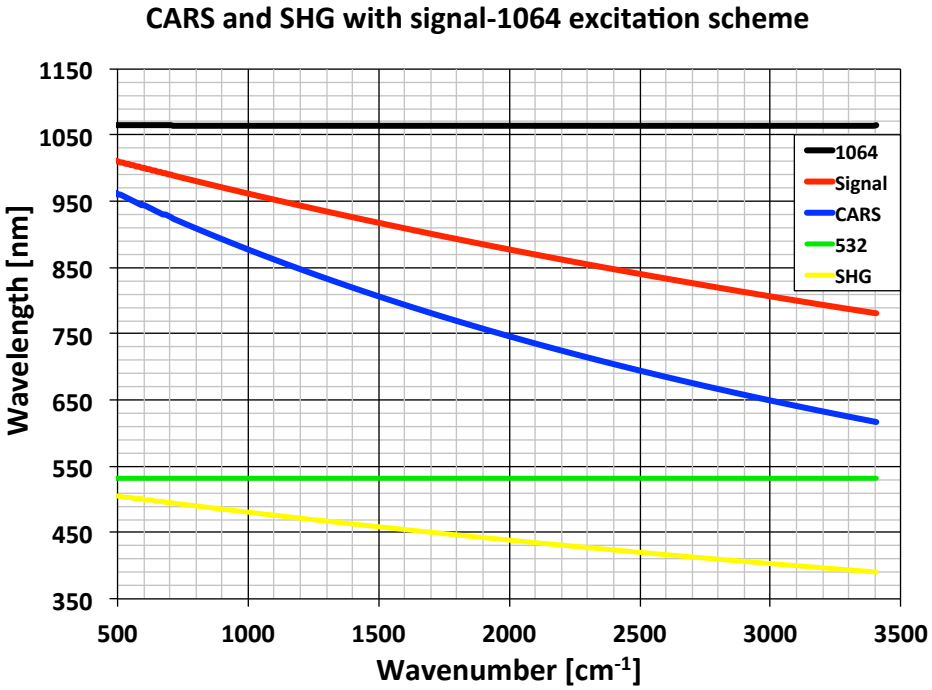

Fig. 3.48 Spectral characteristic of CARS and SHG signals with signal-1064 scheme.

Automation on the wavelength OPO setting and on the spectral measurement

In order to have an automatic scan of the OPO output wavelengths some specific routines have been developed using Labview. Several strategies have been used to set the desired output wavelength and power according to the constraints on the stability of the OPO output power/wavelength and the overall speed of the measurement. More stable OPO output needs a certain waiting time that could be too long for some measurement experiments.

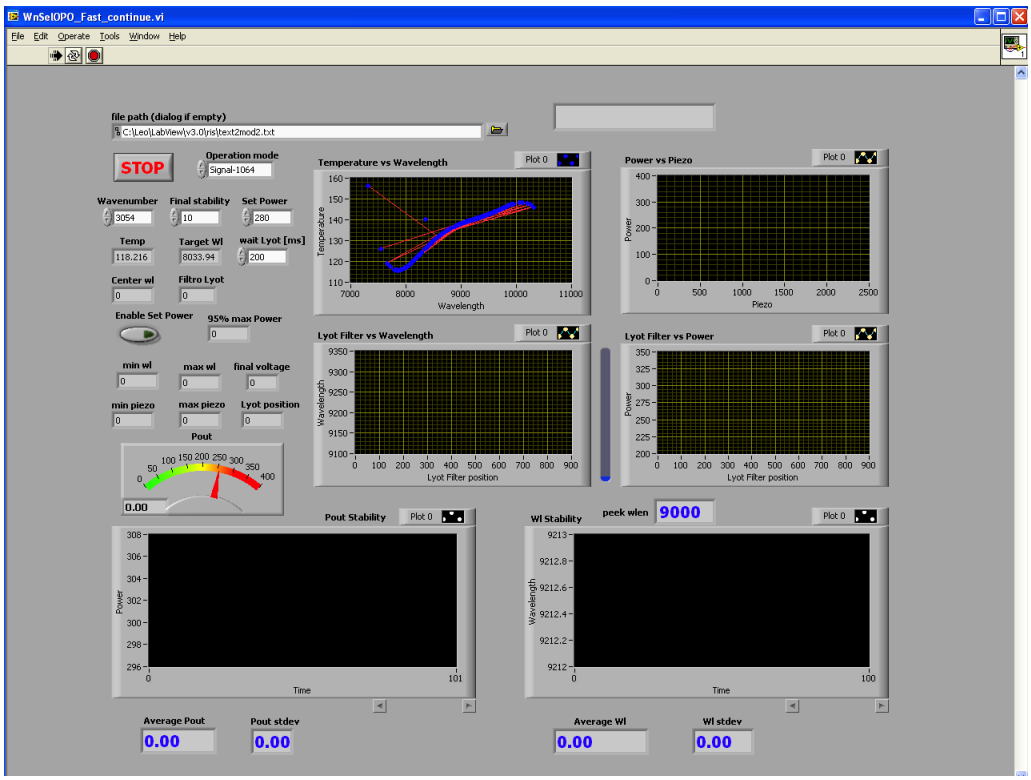

Fig. 3.49 Graphic interface of the control software based on Labview developed to set the OPO wavelengths.

The modular approach of the Labview platform allowed to design a specific routine to set the OPO outputs wavelengths, that has been reused in the other routines designed to perform automatic spectroscopic measurement for CARS and TPEF techniques.

In order to measure CARS spectra or TPEF excitation yield spectra, the OPO outputs wavelengths should be scanned over a selected range with a certain number of intermediate steps at a specified spectral distance from each other. In correspondence of each step a specified number of measurements of the photodetector output voltage are done and the average value together with the relative standard deviation is collected. The voltage measurements are done by means of a digital multimeter (Agilent 34401A). Instantaneous OPO signal power is measured through the internal photodiode of the OPO in arbitrary units.

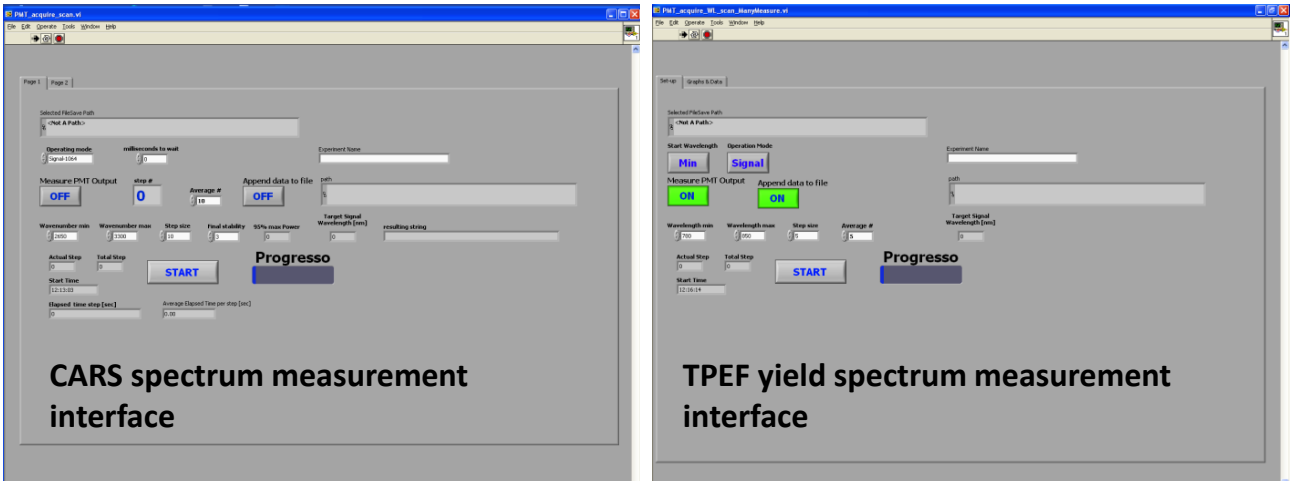

Fig. 3.50 Graphic interface of the control software based on Labview developed to perform automatic CARS (left) and TPEF (right) spectroscopy measurement.

The measured CARS spectra can be weighted by the third power of the instantaneous power or for TPEF by the second power of the instantaneous power, in a way to reduce fluctuation in the spectra due to variation of the excitation power between different spectral points.

A modification of the measurement system will be done adding a direct measurement with a certified power meter of part of the OPO beams deflected by a beam splitter placed before the FV300 scan head optical input, that will be characterized in terms of transmission spectrum over the whole OPO spectral range.

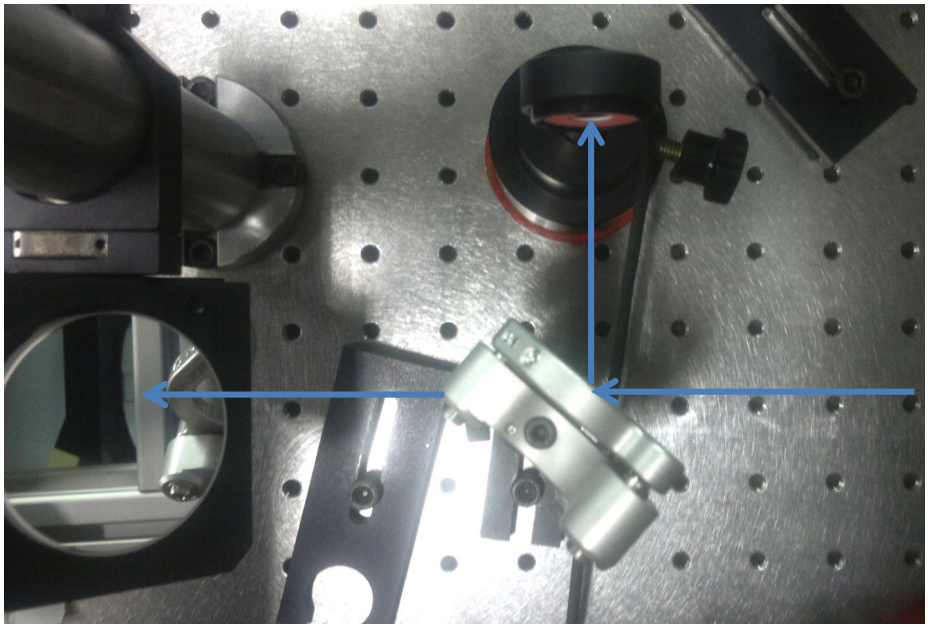

Fig. 3.51 Detail of the optical table where it is shown the beam splitter used to deflect a small part of the excitation beams in order to measure their instantaneous power with a certified power meter.

4. Metrological characterization of the multimodal CARS microscope

Multimodal CARS-SHG-TPEF allows to image with high 3D resolution living samples and it allows to study biological systems better mimicking the "reality" and, in principle, with a quantitative approach.

The measurand is an amount of substance (expressed in submultiples of *mol* according to the International System of units SI) present in the focal volume (expressed in submultiples of m^3 a SI derived units) defined by the optical lenses, transduced in light intensity. In CARS microscopy this represents the number of excited molecular bonds in the focal volume, in SHG microscopy the number of excited non-centrosymmetric molecules and in TPEF microscopy the number of excited fluorophores.

The contrast arising in microscope imaging is then related to the concentration of a target substance that involves two main measurement aspects:

- The amount of substance responsible of the contrast imaging
- The excited focal volume and thus the spatial localization of that amount of substance.

These two aspects are thus related to two different measurement capabilities of the microscope that can be treated as two separate problems in terms of the evaluation of the uncertainty budget.

In this paragraph the study of the uncertainties related to these two simultaneous measurements, is explained together with the strategies that were adopted and that could be adopted in further analysis to obtain an experimental metrological characterization of this complex measurement system.

Amount of substance measurements

The imaging contrast in multimodal microscopy, is directly related to the amount of a target substance, and according to the specific nonlinear optical process exploited, the target substance can be excited emitting a light radiation. This process involves a measurement system composed by several modules, namely:

- The excitation sources
- The optical components to address, optimize and focus the excitation sources on the specimen
- The optical components to collect, filter and optimize the generated signals from the specimen
- The photo detectors to transduce light intensity in electric signals
- The electronic system to amplify, acquire and optimize the electric signals from the detectors
- The software to manage the measurement and process the acquired data.

Each of these modules adds in different ways sources of uncertainty to the final measure. Also the nonlinear processes exploited have some sources of uncertainty that must be considered as for example the non-resonant background in the distortion of the CARS spectrum with respect to the linear Raman spectrum of a substance.

In CARS microscopy the ability to detect the amount of a specific substance is also related to the type of the sample that should be studied. Measuring the amount of substance in solutions with the pure target substance diluted in different concentrations of a pure solvent, is much easier than measuring the amount of the same substance in a mixture of many other chemical species. This because to measure an amount of a specific substance it is needed a micro-spectroscopy analysis, and sometimes it could be a really tough work discriminating the target substance spectrum in the overall measured spectrum. This is a strong limitation together with the presence of the non-resonant background when the measurement of an amount of substance is performed using CARS microscopy. However this does not exclude that in a next future CARS micro-spectroscopy will give the possibility to select a specific substance to image or to determine the chemical composition of a sample.

Some experimental methods can be used to overcome this limitation in a biological sample that is a very complex mixture of many chemical species. One of the most common is the isotope-labelling of the target substance, including a chain-deuterated modification of the molecules, since the symmetric CD_2 stretch band vibration is located around 2100 cm^{-1}, in a spectral region isolated from any Raman band of natural biological molecules [68]. In this case tuning the excitation source in order to excite the vibration around 2100 cm^{-1} is possible to observe only the bonds related to the symmetric CD_2 stretch, minimizing any contribution due to other chemical species present in the sample.

The CARS signal is related to the square modulus of the third-order susceptibility $\chi^{(3)}$ of the sample that is linearly related to the number of molecules N_m excited, also called in literature oscillators:

$$\chi^{(3)} = \frac{N_m A}{\Delta - i\Gamma} + \chi_{nr}^{(3)} \tag{26}$$

Where the square modulus of $\chi^{(3)}$ is given by:

$$\left|\chi^{(3)}\right|^2 = \frac{N_m^2 A^2}{\Delta^2+\Gamma^2} + \frac{2N_m A \chi_{nr}^{(3)}}{\Delta^2+\Gamma^2}\Delta + \left(\chi_{nr}^{(3)}\right)^2 \tag{27}$$

Starting from eq. (9) that represents a simplified case of CARS generation in a slab of a homogeneous material with a thickness L, and expliciting the *detuning* from the Raman resonance frequency Δ, the CARS intensity generated is thus given by:

$$I_{aS}(\omega_{aS}, L) = \frac{9\omega_{aS}^2}{16\varepsilon_0^2 c^4 n(\omega_{aS}) n(\omega_S) n^2(\omega_p)} \left[\frac{N_m^2 A^2}{\left(\omega_R - \omega_p + \omega_S\right)^2 + \Gamma^2} + \frac{2N_m A \chi_{nr}^{(3)}}{\left(\omega_R - \omega_p + \omega_S\right)^2 + \Gamma^2}\left(\omega_R - \omega_p + \omega_S\right) + \left(\chi_{nr}^{(3)}\right)^2\right] I^2(\omega_p, 0) I(\omega_S, 0) L^2 \frac{\sin^2\left(\frac{1}{2}\Delta k L\right)}{\left(\frac{1}{2}\Delta k L\right)^2} \tag{28}$$

From this relation it can be noted that the CARS signal intensity depends quadratically with the number of molecules excited if the true value of the non-resonant background term of the third-order susceptibility of the material $\chi_{nr}^{(3)}$ becomes negligible.

It is also possible to note that the sources of uncertainty can be divided in two main groups that are:

- Those related to the type of the sample where its chemical and optical characteristics play a role.
- Those related to the experimental set-up in which the different modules of the measurement system play a role.

For such regards the type of sample, as it was previously described, the complexity of the CARS spectrum of the target molecule with respect to the other molecules present in the sample is one of the first limitation

on the measurement of the amount of that substance in the sample. This limitation is a common problem also for the traditional Raman spectroscopy/micro-spectroscopy that was investigated from long time also placing the fundamentals for a specific discipline called chemometrics that studies possible methods and algorithms (such as for example: Principal Component Analysis (PCA), Independent Component Analysis (ICA), Hierarchical Cluster Analysis (HCA), etc.) to process high amount of data searching inside the measured spectra for specific regressions, patterns or clusters, related to the target species analysed according to the strategy used.

A method based on the maximum entropy model was proposed by E. Vartiainen et al. [69] to retrieve the maximum entropy (ME) phase of CARS spectra and extract the imaginary part of the resonant CARS signal. This process allows retrieving the effective Raman spectra from the measured CARS spectra, with quite identical resulting spectra to those obtained using linear Raman spectroscopy (Fig. 4.1).

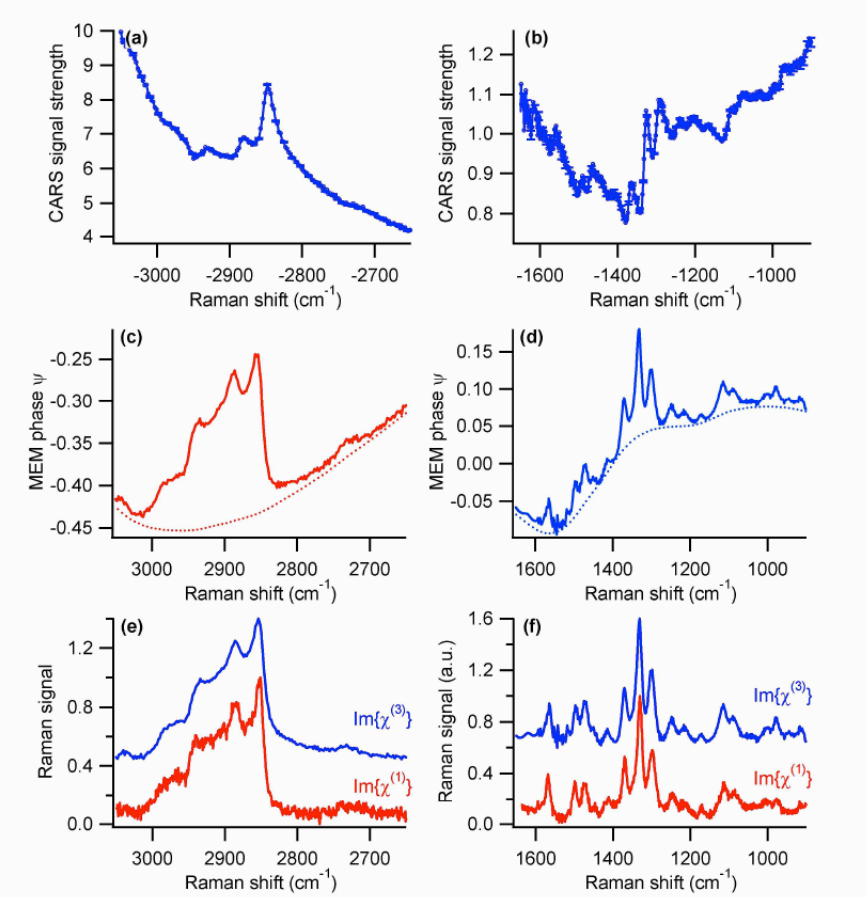

Fig. 4.1 The CARS line-shapes in the vicinity of vibrational resonances of DMPC (a) and ADP/AMP/ATP (b). The corresponding ME phases, $\psi(\omega)$, (solid lines) and the estimated background phase, $-\varphi_{est}(\omega)$, (dotted lines) of DMPC (c) and ADP/AMP/ATP (d). The Raman line-shapes obtained from CARS spectra (blue lines) and the corresponding spontaneous Raman scattering spectra (red lines) of DMPC (e) and ADP/AMP/ATP (f) (from [69]).

Another source of uncertainty that can be deducted from eq. (28), is the variation of the refractive indexes of the sample with respect to the position in the sample, to the wavelengths and the polarizations used. In biological samples changes of the refractive index between different tissues, cell compartments, cells types is quite typical and generally has a value in the range between 0.9 and 1.45 [70-73]. These changes in the refractive index along the excitation signals propagations add also some phase differences that can affect the phase matching condition. This phenomenon could be maximized or minimized according to the

numerical apertures of the lenses used to focus the excitation sources on the sample, that determine the possible propagation angles of the incident radiations. Increasing the number of possible incident angles could help to minimize this phenomenon.
In the simplified eq. (28) it is also considered the thickness of the homogeneous material and how it was explained previously, this dimension plays a crucial role depending on what direction is used to detect the CARS signal. When the epi-detection is used, the interference phenomena that arise due to the oscillating satisfaction of the phase matching condition must be also considered to measure the amount of target specie. This is also a problem linked with the spatial resolution of the CARS microscope and thus with the optical lenses used to focus the excitation sources on the sample, since epi-detection is optimal when scatterers have sizes much smaller than the excitation wavelengths Fig 2.3.
The sample could also absorb or scatter part of the excitation and emitted signals depending of its thickness and transmittance, attenuating the generated CARS signal also in non-uniform way if the sample is not homogeneous, adding a further source of uncertainty.

$$I_{aS}(\omega_{aS}, L) = \Omega_{sp,aS} \frac{9(2\omega_p-\omega_S)^2}{16\varepsilon_0^2 c^4 n_{aS} n_S n_p^2} \left[\frac{N_m^2 A^2}{(\omega_R-\omega_p+\omega_S)^2+\Gamma^2} + \frac{2N_m A \chi_{nr}^{(3)}}{(\omega_R-\omega_p+\omega_S)^2+\Gamma^2} (\omega_R-\omega_p+\omega_S) + \left(\chi_{nr}^{(3)}\right)^2 \right] [\Omega_{sp,p} I_p]^2 \Omega_{sp,S} I_S L^2 \frac{\sin^2\left(\frac{1}{2}\Delta kL\right)}{\left(\frac{1}{2}\Delta kL\right)^2} \quad (29)$$

In the eq. (29) are also reported the attenuation factors $\Omega_{sp,aS}$, $\Omega_{sp,p}$ and $\Omega_{sp,S}$ due to the sample absorption referred to the three different wavelengths in CARS process.

For such regards the experimental set-up of the measurement system the instabilities of the intensities (I_p and I_S) and those of the wavelengths (ω_p and ω_S) of the excitation sources could affect dramatically the intensity of the generated CARS signal as can be deducted from eq. (29). It is then very important having very stable excitation sources and it is a good practice to record their instantaneous intensities and wavelengths during the CARS experiments.

The optical components used to address and optimize the excitation sources on the sample, such as mirrors, optical filters, iris, spatial filters and lenses, attenuate the excitation sources in function of the wavelength. These attenuations could be expressed using two independent attenuation factors for the pump and Stokes signals, respectively Ω_p and Ω_S that lead to different effective intensities for the pump and Stokes signals at the sample:

$$I_p = \Omega_p I_{p,0} \quad I_S = \Omega_S I_{S,0} \quad (30), (31)$$

The CARS signal generated at the sample is then collected by the same excitation lens if it is epi-detected or by a condenser lens if it is forward detected. The collected signal is then reflected with mirrors, optically filtered and in forward detection focused with a lens on the sensitive surface of a photodetector. All these passages could attenuate the CARS signal and it is also possible that a very little part of the excitation sources could be also detected together with the CARS signal. Together with the CARS signal some other radiations such as two-photon excited autofluorescence could be generated from the sample in the same CARS spectral window. Also this condition that could arise with low probability if infrared excitation sources are used, should also be also taken into account. Moreover since the CARS signal could be very weak in certain condition it is very important to prevent the ambient light to be detected together with the CARS signal. With this purpose a shield in aluminium has been designed and realized to cover the microscope stage and part of the optical table where the detectors for forward detection are placed. The CARS intensity

before the detector considering all these phenomena could be expressed using the following simplified formula:

$$I_{aS,det} = \Omega_{tr,as} I_{as} + \Omega_{tr,p} \Omega_{sp,p} \Omega_p I_{p,0} + \Omega_{tr,S} \Omega_{sp,S} \Omega_S I_{S,0} + I_{amb} + \Omega_{af} I_{af} \quad (32)$$

Where $\Omega_{tr,as}$ represents the attenuation factor of the generated CARS signal at the sample I_{as} due to the optical components between the sample and the detector, $\Omega_{tr,p}$ and $\Omega_{tr,S}$ represent respectively the attenuation factors of the pump and stokes signals that are transmitted through the sample due to optical components between the sample and the detector, I_{amb} is the residual ambient light that could impinge the detector surface and $\Omega_{af} I_{af}$ is the attenuated two-photon excited autofluorescence signal that could impinge the detector surface.

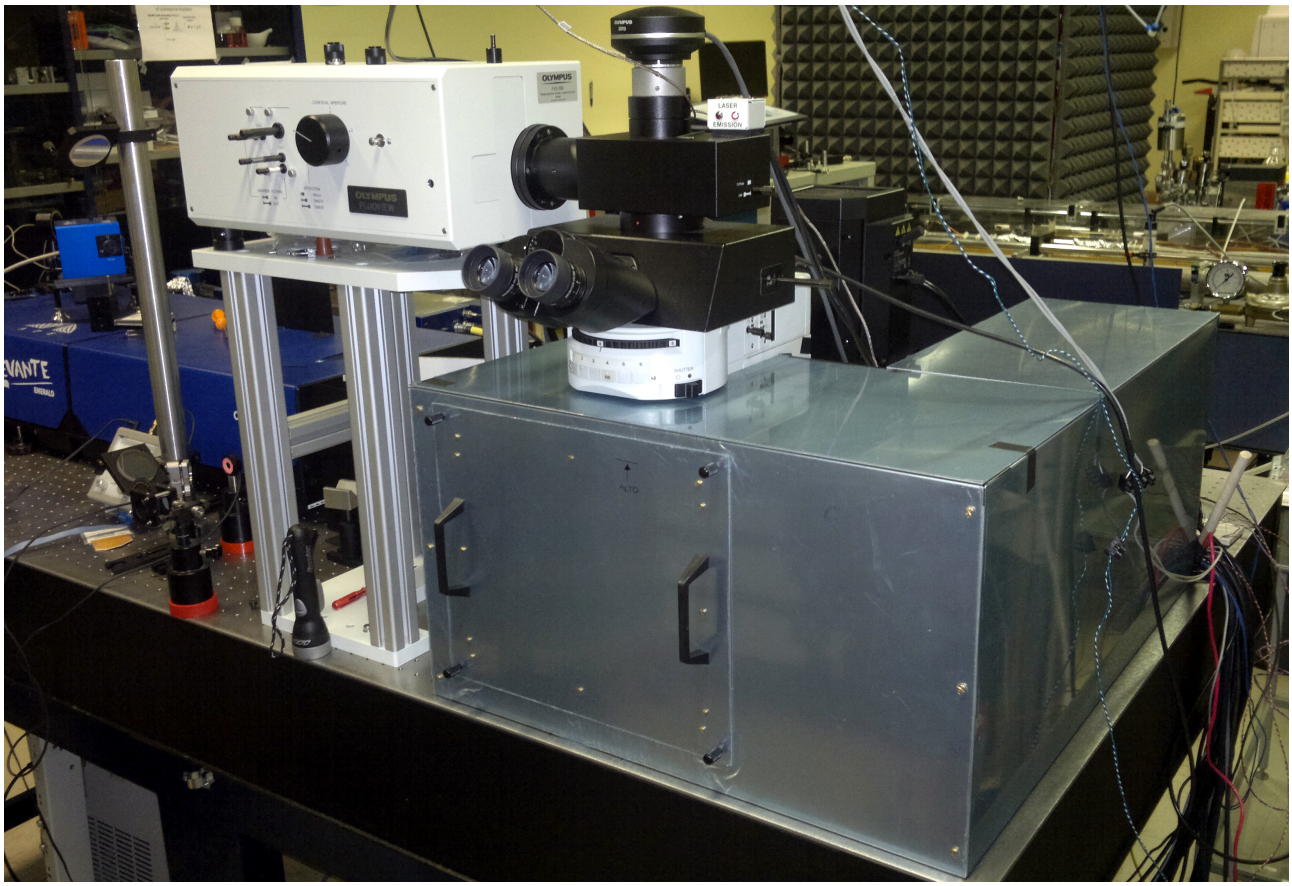

Fig. 4.2 Multimodal CARS-SHG-TPEF microscopy realized at INRIM with the aluminum shield to protect measurements from the ambient stray light.

The detector converts the light intensity on its surface in an electric current according to its transfer function. The detector's current is pre-amplified with a transimpedance amplifier that converts the input current in an output voltage. This transimpedance amplifier has the possibility to adjust the amplifier gain and trim an offset voltage in order to optimize the image contrast. Moreover the gain of a PMT depends on the voltage applied to the dynodes that could be also modified to change the PMT sensitivity. The amplified output voltage can be expressed as:

$$V_{aS,det} = I_{aS,det} Q G_{amp} + V_{offset} \quad (33)$$