Biophotonics course
Exam of February 22, 2010
Total duration: 3h (part I: 1h30, part II: 45 min, part III: 45 min)
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Please use three different exam sheets for parts I, II and III.
Answers in English or in French.

Part I (A. Alexandrou)
Fluorescence Resonant Energy Transfer (FRET)
All questions can be answered in a few lines each.

In FRET, energy transfer takes place between a donor fluorophore D and an acceptor fluorophore A. The efficiency E of the energy transfer is defined as the probability for the excited donor to return to its ground state by energy transfer to the acceptor.

1) Based on this definition, give the equation relating the energy transfer efficiency E to the rate of all radiative and non-radiative donor decay processes in the absence of the acceptor, k_D, and to the rate of energy transfer to the acceptor, k_T.
2) Derive from the above equation, the equation relating the energy transfer efficiency E to the excited-state lifetime of the donor in the absence of the acceptor, τ_D, and to the excited-state lifetime of the donor in the presence of the acceptor τ_{D_A}.
3) Indicate how the energy transfer efficiency E can be expressed in terms of the donor emission intensity in the absence of the acceptor, I_D, and of the donor emission intensity in the presence of the acceptor, I_{D_A}.
4) What are the possible sources of error when the energy transfer efficiency E is determined experimentally by measuring I_D, I_{D_A} and using the equation derived in point 3) ?
5) Why is it preferable to determine the energy transfer efficiency E by measuring τ_D and τ_{D_A} and using the equation derived in point 2)?
6) Both the formulae derived in points 2) and 3) require measurements in two kinds of samples, one with and one without acceptor. Derive a formula for the determination of the energy transfer efficiency E that requires only measurements with the donor-acceptor sample using the donor emission intensity in the presence of the acceptor, I_{D_A}, and the acceptor emission intensity in the presence of the donor, I_{A_D}.
7) Usually the detector used to measure the donor and acceptor emission has a wavelength-dependent detection efficiency, i. e. the detection efficiency at the donor emission wavelength, η_D, is different from the detection efficiency at the acceptor emission wavelength, η_A. Introduce the necessary modification of the previous formula to take this into account.

8) The rate of energy transfer, k_T, can be expressed in terms of the donor-acceptor distance R and of the constant R_0 depending on the spectral properties of the fluorophores as well as their relative orientation by the equation:

$$k_T = k_D \left(\frac{R_0}{R} \right)^6 .$$

Derive the expression of the energy transfer efficiency E as a function of R and R_0.

9) Consider the case of energy transfer between one donor and n acceptors. Give the expression of the energy transfer E as a function of k_T and k_D and as a function of R and R_0.

10) What biologically relevant information can be obtained using FRET experiments?

Single-pair FRET

11) Consider the case of a single-molecule observation of a single donor-acceptor pair using a wide-field microscope and a CCD camera. A sequence of 2D images is acquired using an interference filter to select the donor emission and the signal of the donor emission is determined for each image. At a certain time point, the acceptor undergoes a stepwise photobleaching and the donor emission signal changes from S to S'. Determine the energy transfer efficiency E. Assuming R_0 is known, determine the donor-acceptor distance R.

We will now consider a two-color confocal setup which detects bursts of donor and acceptor emission as donor-acceptor pairs cross the focal volume (see figure 1).

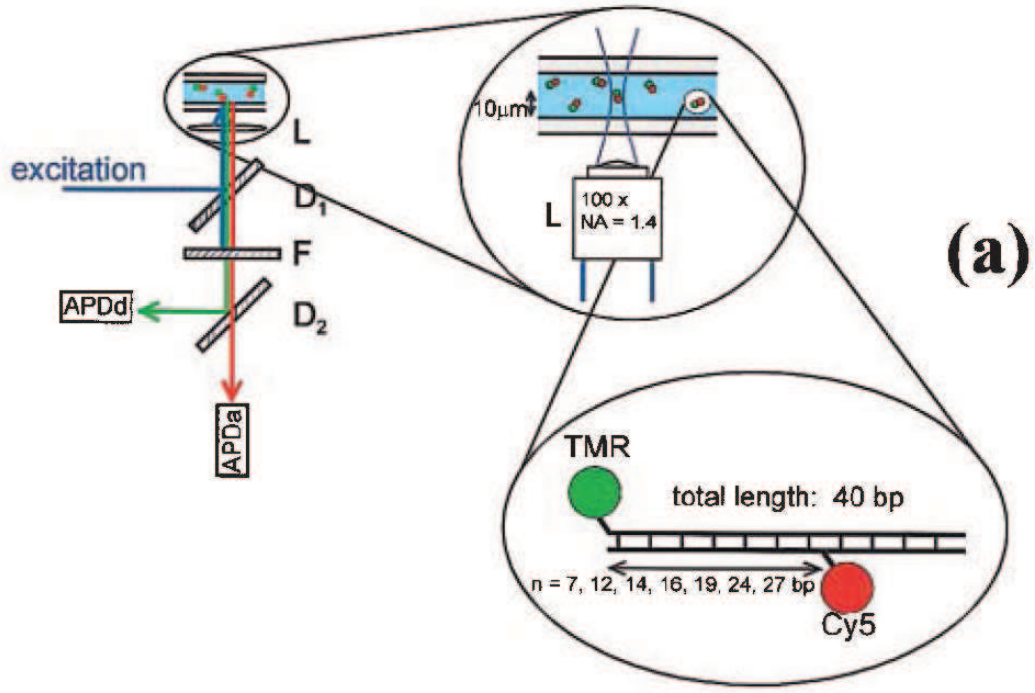

Figure 1. Two-color confocal setup. L: lens, D1 and D2: dichroïc mirrors, F: filter rejecting the excitation light, APDa and APDb: avalanche photodiodes detecting the donor (Tetramethylrhodamine, TMR) and acceptor (Cy5) emission, respectively. Blue beam: laser excitation of the donor, green: donor emission, red: acceptor emission.

12) Explain how this setup eliminates light emitted from donor-acceptor pairs located outside of the focal volume. (The limited size of the APD detectors renders the use of a pinhole superfluous.)
13) Give a *qualitative* schematic of the signal expected from the two APD detectors in the following cases: (i) donor-only sample, (ii) acceptor-only sample, (iii) donor-acceptor sample with donor and acceptor 16 DNA base pairs apart, and (iv) donor-acceptor sample with donor and acceptor 24 DNA base pairs apart. One base pair corresponds to 0.34 nm of length along the DNA strand. $R_0 = 6.5\,\text{nm}$ for the TMR-Cy5 donor-acceptor pair. (Calculate the energy transfer efficiency for the cases (iii) and (iv).)
14) Draw again the expected signal in cases (i) and (ii) of the previous question after taking into account two common problems in FRET measurements: a) leakage of donor emission into the acceptor detection channel and b) direct (not via FRET) excitation of the acceptor with the donor excitation laser.
15) In most cases, it is not possible to have a 100% labeling efficiency. This means that, in any donor-acceptor sample, acceptor-only and donor-only molecules will also be present. Based on your observations in the cases (ii)-(iii) and (i)-(iv) discussed in the two previous questions, comment on the difficulties encountered when trying to measure high or low energy transfer efficiencies.

In order to overcome these difficulties, the experimental setup shown in Figure 2 was proposed (A. N. Kapanidis et al, Proc. Natl. Acad. Sci. USA, **101** 8936–8941 (2004) and N. K. Lee et al., Biophys. J. **88**, 2939–2953 (2005)). This approach was termed ALEX-FRET (alternating laser excitation FRET). Its difference from the previous setup is that the donor and the acceptor are excited alternatively using two different excitation lasers (a 514-nm laser for donor excitation and a 638-nm laser for acceptor excitation). Electro-optic modulators allow switching on and off of the two lasers in a way that the sample is alternatively excited with the donor and with the acceptor excitation laser. Four signals are recorded in this case: donor emission upon donor excitation, $f_{D_{exc}}^{D_{em}}$, donor emission upon acceptor excitation, $f_{A_{exc}}^{D_{em}}$, acceptor emission upon donor excitation, $f_{D_{exc}}^{A_{em}}$, and acceptor emission upon acceptor excitation, $f_{A_{exc}}^{A_{em}}$.

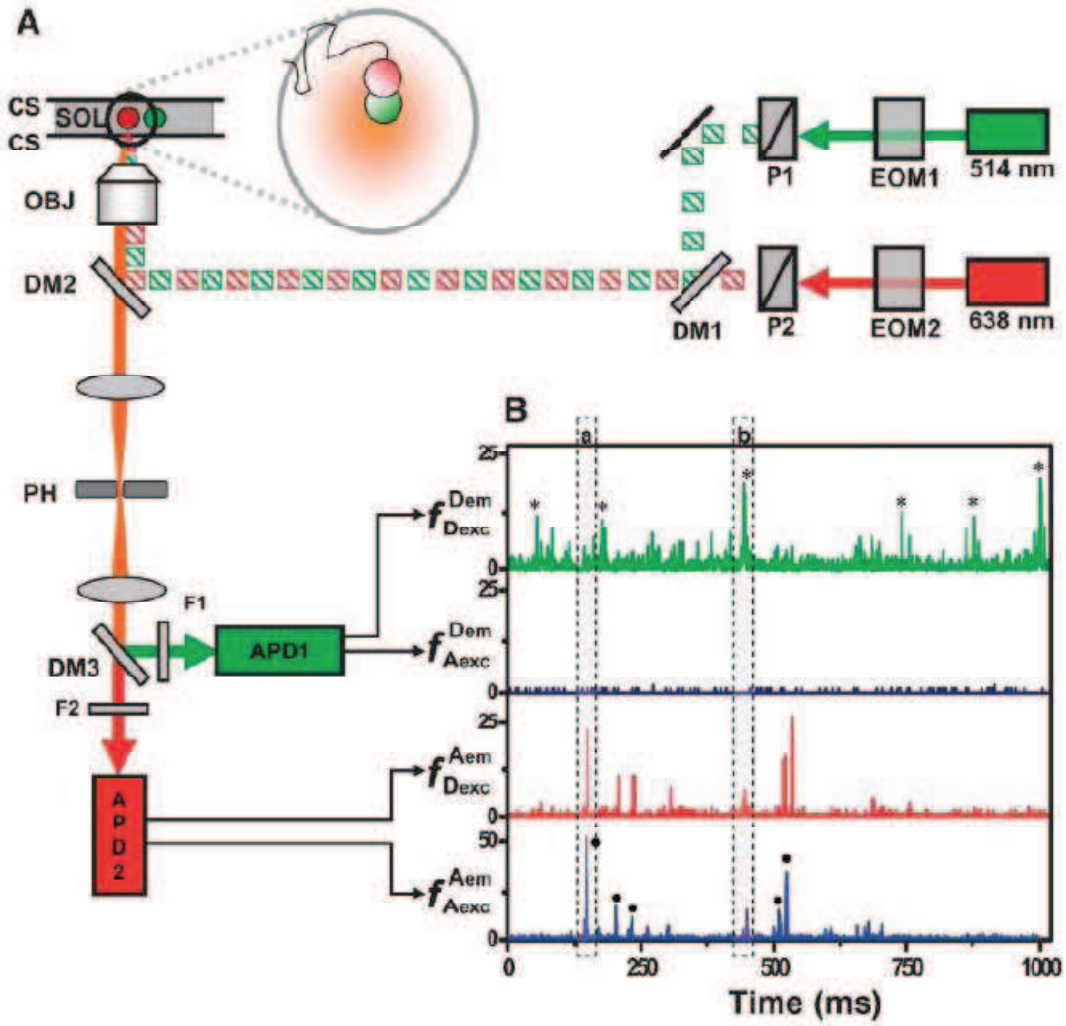

Figure 2. Alternating laser excitation FRET. EOM1, EOM2: electro-optic modulators, P1, P2: polarizers, DM1, DM2, DM3: dichroïc mirrors, OBJ: objective, CS: coverslip, SOL: solution, PH: pinhole, F1, F2: filters for donor and acceptor emission, respectively, APD1, APD2 avalanche photodiodes for detection of donor and acceptor emission, respectively. Modulators combined with polarizers result in alternating laser excitation.

16) Using this modified setup, one can distinguish between the cases: donor-only, acceptor-only, high FRET efficiency, low FRET efficiency. Which case do the signals in the dashed rectangle labeled a correspond to? Which case do the signals labeled with a * correspond to?
17) The FRET efficiency can be calculated using the equation of question 7). In addition, a stoichiometry parameter S can be defined as:

$$S = \frac{f_{D_{exc}}}{f_{D_{exc}} + f_{A_{exc}}},$$

where $f_{D_{exc}} = f_{D_{exc}}^{A_{em}} + \frac{q_A \eta_A}{q_D \eta_D} f_{D_{exc}}^{D_{em}}$ and $f_{A_{exc}} = f_{A_{exc}}^{A_{em}} + f_{A_{exc}}^{D_{em}}$. Show that this stoichiometry parameter is independent of the donor-acceptor distance. Show that both the FRET efficiency *E* and the stoichiometry parameter *S* vary between 0 and 1.
18) Two-dimensional plots of the FRET efficiency *E* and the stoichiometry parameter *S* can be shown (see Figure 3). Indicate in this two-dimensional plot the areas corresponding to the four cases mentioned above: donor-only, acceptor-only, high

FRET efficiency, low FRET efficiency. Based on this plot, comment on how the ALEX-FRET scheme can be used for accurate FRET measurements avoiding artefacts.

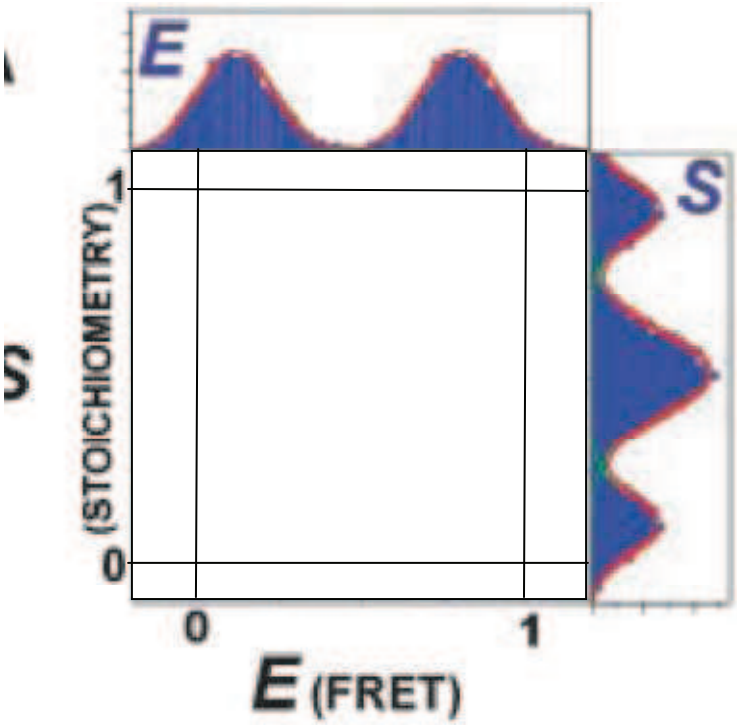

Figure 3. Two-dimensional plot of the FRET efficiency E and the stoichiometry parameter S.

Biophotonics Examination – February 22nd, 2010

Duration : 45 min (5 points)

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Biochips, fluorescent biochips, DNA biochips, SPR biochips (Benisty Henri)

1) What are the typical spot diameters and spot center-to-center distances in a biochip ?

2) What is the typical length of DNA probes grafted inside the spots

3) How many different combinations of DNA single-strands are there for 10 nucleotides (reminder 2^10=1024~10^3). Can they all be put on a standard biochip format ?

4) The targets are labelled by Cy3 and Cy5. Remind the wavelengths and laser excitations of each.

5) What can be for example a difference between Cy3 and Cy5 labelled probes in a differential expression experiment ?

6) Why is it much preferable to use the RATIO of Cy3 and Cy5 fluorescence, rather than an their absolute fluorescent measurement ?

7) Illustrate by a very simple drawing a covalent grafting (as obtained after silanization, spotting and washing, before use of the DNA chip)

8) Scanner confocality and collection :

(a) What is the fraction of a fluorophore emission captured into NA=0.5 in air if the fluorophore is in air

(b) Why not using the highest NA (~0.9) but only NA<0.6 when scanning real slides ?

9) Surface plasmon resonance :

(a) Is it possible to sustain a plasmon at an ordinary interface between two dielectrics

(a) Confirm your answer by drawing the schematic profile of the field of a surface plasmon at a gold/water interface for instance

(b) What is the effect of the index change at the surface, when proteins adsorb onto the functionalized Au layer ?

(c) Why is a prism needed to couple light to the plasmon ?

EXAM

45 min (5 points)

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A schematic diagram of a simplified time-domain OCT system is shown in Fig. 1.

☞ Explain how cross-sectional (xz-oriented) images of the sample are obtained.

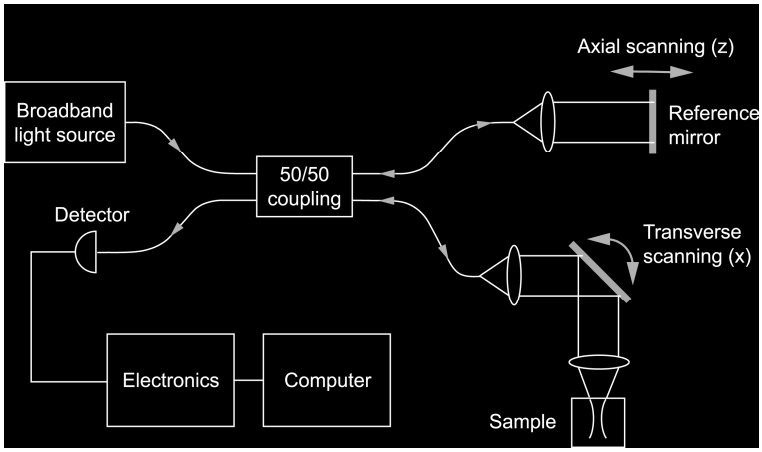

Fig. 1: Schematic of the time-domain OCT system

We consider the simplified schematic of the Michelson interferometer shown in Fig. 2, where the sample has been replaced by a perfectly reflecting mirror.

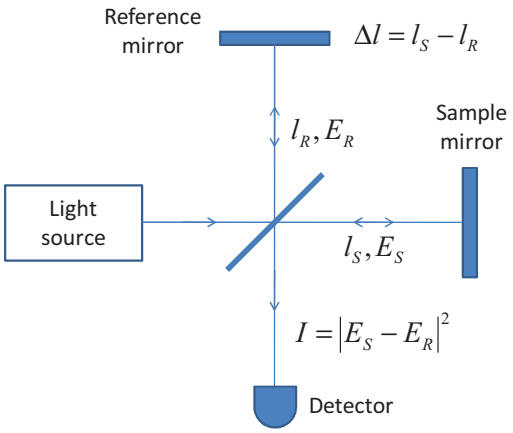

Fig.2: The Michelson interferometer.

Interferometer with coherent light

If the light source is monochromatic (frequency ω), reflection from the reference and sample mirrors produces two electric field components E_R and E_S at the detector:

$$E_R = A_R exp[-j(2k_R l_R - \omega t)] \quad \text{and} \quad E_S = A_S exp[-j(2k_S l_S - \omega t)].$$

The optical intensity detected by the photodetector is proportional to $I = |E_R + E_S|^2$.

☞ Show that all the interferometric information is contained in the real part of the cross-spectral term $E_S E_R^*$.

We suppose that we are in free space. We have then $k_R = k_S = 2\pi/\lambda$, where λ is the optical wavelength.

☞ Express the interferometric signal at the photodetector $I = real\{E_S E_R^*\}$ as a function of A_S, A_R, λ and Δl, where $\Delta l = l_S - l_R$ is the length mismatch in the interferometer arms.

Interferometer with low coherence light

A low coherence light source consists of a finite bandwidth of frequencies rather than just a single frequency. The reference and sample fields are then functions of frequency:

$$E_R(\omega) = A_R(\omega) exp\{-j[2k_R(\omega)l_R - \omega t]\} \quad \text{and} \quad E_S(\omega) = A_S(\omega) exp\{-j[2k_S(\omega)l_S - \omega t]\}.$$

The interference signal at the photodetector is:

$$I = real\left\{\int_{-\infty}^{+\infty} E_S(\omega)\, E_R(\omega)^* \frac{d\omega}{2\pi}\right\}$$

The power spectrum of the light source $S(\omega)$ and the phase mismatch $\Delta\phi(\omega)$ are defined as:

$$S(\omega) = A_S(\omega)A_R(\omega)^* \quad \text{and} \quad \Delta\phi(\omega) = 2k_S(\omega)l_S - 2k_R(\omega)l_R.$$

☞ Express the interference signal I as a function of $S(\omega)$ and $\Delta\phi(\omega)$.

We consider the case where the sample and reference arms consist of a uniform, linear non dispersive medium. Let the spectrum of the light be band-limited with a center frequency of ω_0. Using a first-order Taylor expansion around the center frequency, we can write

$$k_S(\omega) = k_R(\omega) = k(\omega_0) + k'(\omega_0)(\omega - \omega_0).$$

☞ Show that the interferometric signal I consists of a carrier and an envelope. Show that the carrier oscillates with increasing path length $2\Delta l$ at a frequency of $k(\omega_0)$. Show that the envelope is essentially the inverse Fourier transform of the source spectrum $S(\omega)$.

☞ Rewrite the interferometric signal I with the following parameters:

- the center frequency phase velocity : $v_p = \omega_0/k(\omega_0)$
- the group velocity : $v_g = 1/k'(\omega_0)$

Assume that the light source has a Gaussian power density given by

$$S(\omega) = exp\left[-\frac{(\omega - \omega_0)^2}{2\sigma_\omega^2}\right]$$

☞ Express the interference signal I at the photodetector. Show that the envelope of the interferometric signal is a Gaussian with width $\sigma_t = 1/\sigma_\omega$.

The axial resolution of an OCT system is defined as the width of the envelope in units of length mismatch Δl, for propagation in free space. Note that in free space both the phase velocity and the group velocity equal the speed of light c.

☞ Show that the standard deviation axial resolution is $\Delta l_{SD} = \frac{c}{\sigma_\omega}$

For a Gaussian with standard deviation σ, the full-width at half-maximun is $2\sigma\sqrt{2ln2}$.

☞ Express the FWHM axial resolution Δl_{FWHM} of an OCT system as a function of the FWHM wavelength bandwidth $\Delta\lambda$ and center wavelength λ_0.

We assume now that the light source has a rectangular-shaped power density represented in Fig. 3. The center frequency is $\omega_0 = \frac{\omega_1+\omega_2}{2}$ and the frequency bandwidth is $\Delta\omega = \omega_2 - \omega_1$.

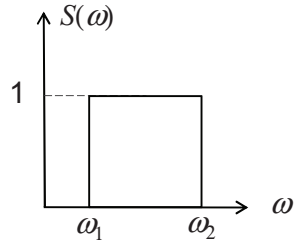

Fig.3: Rectangular-shaped spectrum.

☞ Express the interference signal at the photodetector.

☞ What is then the FWHM axial resolution Δl_{FWHM} of an OCT system as a function of the wavelength bandwidth $\Delta\lambda$ and center wavelength λ_0 ?

☞ Suppose that the axial resolution Δl_{FWHM} is the same using an illumination source with a Gaussian-shaped spectrum or a rectangular-shaped spectrum. Are both spectra equivalent for OCT imaging ? Why ?

Note: The Fourier transform of $f(\sigma) = \exp\left(-\frac{\sigma^2}{2a^2}\right)$ is $\tilde{f}(\delta) = \exp\left(-\frac{\delta^2}{2\delta_L{}^2}\right)$, with $\delta_L = \frac{1}{2\pi a}$.

EXAM (correction)

☞ Show that all the interferometric information is contained in the real part of the cross-spectral term $E_S E_R^*$.

$$I = |A_R|^2 + |A_S|^2 + 2real\{E_S E_R^*\}$$

☞ Express the interferometric signal at the photodetector $I = real\{E_S E_R^*\}$ as a function of A_S, A_R, λ and Δl, where $\Delta l = l_S - l_R$ is the length mismatch in the interferometer arms.

$$real\{E_S E_R^*\} = A_R A_S \cos\left(4\pi \frac{\Delta l}{\lambda}\right)$$

Interferometer with low coherence light

☞ Express the interference signal I as a function of $S(\omega)$ and $\Delta\phi(\omega)$.

$$I = real\left\{\int_{-\infty}^{+\infty} S(\omega)\, e^{-j\Delta\phi} \quad \frac{d\omega}{2\pi}\right\}$$

☞ Show that the interferometric signal I consists of a carrier and an envelope. Show that the carrier oscillates with increasing path length $2\Delta l$ at a frequency of $k(\omega_0)$. Show that the envelope is essentially the inverse Fourier transform of the source spectrum $S(\omega)$.

☞ Rewrite the interferometric signal I with the following parameters:

- the center frequency phase velocity : $v_p = \omega_0/k(\omega_0)$
- the group velocity : $v_g = 1/k'(\omega_0)$

$$I = real\left\{e^{-j\omega_0 2\Delta l/v_p} \times \int_{-\infty}^{+\infty} S(\omega-\omega_0)\, e^{-j(\omega-\omega_0)2\Delta l/v_g} \frac{d(\omega-\omega_0)}{2\pi}\right\}$$

Wiener – Khintchin theorem: the autocorrelation function is equal to the inverse Fourier transform of the power spectral density.
The envelope is essentially the inverse Fourier transform of the source power spectrum

Assume that the light source has a Gaussian power density given by

$$S(\omega) = exp\left[-\frac{(\omega-\omega_0)^2}{2\sigma_\omega^2}\right]$$

☞ Express the interference signal I at the photodetector. Show that the envelope of the interferometric signal is a Gaussian with width $\sigma_t = 1/\sigma_\omega$.

$$I = exp\left(-\frac{2\Delta l^2}{{v_g}^2\sigma_t^2}\right)\cos(2\omega_0\Delta l/v_p)$$

☞ Express the FWHM axial resolution Δl_{FWHM} of an OCT system as a function of the FWHM wavelength bandwidth $\Delta\lambda$ and center wavelength λ_0.

$$\Delta l_{FWHM} = \frac{2ln2}{\pi}\left(\frac{{\lambda_0}^2}{\Delta\lambda}\right)$$

We assume now that the light source has a rectangular-shaped power density represented in Fig. 3. The center frequency is $\omega_0 = \frac{\omega_1+\omega_2}{2}$ and the frequency bandwidth is $\Delta\omega = \omega_2 - \omega_1$.

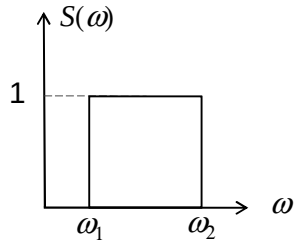

Fig.3: Rectangular-shaped spectrum.

☞ Express the interference signal at the photodetector.

$$I = sinc\left(\Delta l\, \Delta\omega / v_g\right) \cos\left(2\omega_0 \Delta l\, / v_p\right)$$

☞ What is then the FWHM axial resolution Δl_{FWHM} of an OCT system as a function of the wavelength bandwidth $\Delta\lambda$ and center wavelength λ_0 ?

$$sinc(x) = \frac{1}{2}\ for\ x \approx 1.9$$

$$\Delta l_{FWHM} \approx 0.6 \frac{{\lambda_0}^2}{\Delta\lambda}$$

☞ Suppose that the axial resolution Δl_{FWHM} is the same using an illumination source with a Gaussian-shaped spectrum or a rectangular-shaped spectrum. Are both spectra equivalent for OCT imaging ? Why ?

Same resolution but different axial point-spread functions (PSF). The Gaussian-shaped spectrum is the most appropriate because no side lobes in the interferogram envelope.

Note: The Fourier transform of $f(\sigma) = \exp\left(-\frac{\sigma^2}{2a^2}\right)$ is $\tilde{f}(\delta) = \exp\left(-\frac{\delta^2}{2{\delta_L}^2}\right)$, with $\delta_L = \frac{1}{2\pi a}$.