Imaging Biological Systems

Optics Tutorial - Basics of photonic and atomic microscopies

12th OCTOBER 2020

Objective of the session: understand the principles of microscopy and the specificities of each of the techniques presented during the practical: Epifluorescence, TIRF, confocal and atomic force microscopy.
In particular, the following notions will be addressed and should be discussed in the report:

- Magnification, Field of view, focal distances
- Resolution and numerical aperture, pinhole
- Nyquist
- Fluorescence spectra, filters, dichroics
- ...

Teacher:
Antoine Le Gall, legall@cbs.cnrs.fr

Ressources:

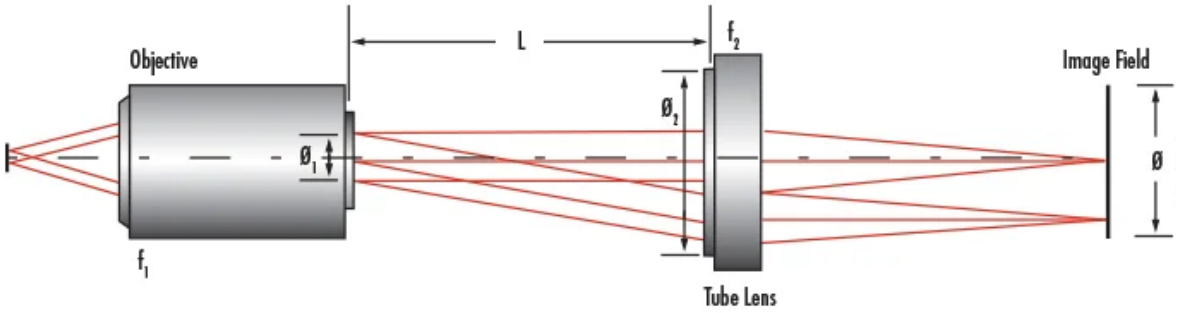
- https://www.edmundoptics.fr/knowledge-center/#!&CategoryId=219
- http://www.iscopecalc.com/
- https://camera.hamamatsu.com/jp/en/camera_simulation_engine/index.html
- https://www.fpbase.org/
- https://www.olympus-lifescience.com/fr/microscope-resource/primer/techniques/technojava/
- https://micro.magnet.fsu.edu/
- https://depts.washington.edu/vurchin/index.php?view=micmeas
- https://myscope.training/#

Part 1 : Choosing the right microscope illumination mode

Robert wants to image a biological sample on his microscope. The sample contains chemically fixed tissues (200 µm in size) on which he wishes to observe fluorophore-labeled genomic loci. The microscope allows to observe the sample either in epifluorescence mode, in TIRF or in confocal mode. Before passing the sample on the microscope, Robert wishes to know :

- if it is possible for him to visualize the integrity of the tissues in one image
- with what resolution he will be able to observe the structures formed by the fluorescent labels.
- what will be the most suitable excitation mode for the sample?

1- Field of view size



Robert's microscope objective is a Nikon with magnification M = 100X. Knowing that the focal length of the tube lens of a Nikon microscope is f2 = 200mm:

1.1.1 Calculate the focal length f1 of the objective :

1.1.2 Calculate the Objective Exit Pupil Diameter ⌀1 knowing that the objective has a numerical aperture of NA=1.45 and requires an oil immersion medium (Oil Refractive Index n_oil = 1.512)

The diameter of the entrance pupil of the tube lens is ⌀2 = 32 mm and the distance between the objective and the tube length is L=200 mm :

1.1.3 Calculate the maximum size of the observation field (Field of View) ⌀ without vignetting.

$$L = \left(\frac{\emptyset_2 - \emptyset_1}{\emptyset}\right) f_2$$

1.1.4 What is the maximum field of view size this represents in the sample plane?

2- If acquisition in epifluorescence

If Robert chooses to make his acquisitions in epifluorescence mode then he has an available emCCD camera whose pixel size is 16 µm and whose sensor size is 13.3 x 13.3 mm.

1.2.1 Given the sensor size and the maximum field of view size calculation performed in the previous section, what will be the limiting factor to observe the sample? Will Robert be able to observe his tissues in their entirety?

1.2.2 Compute the microscope resolution if the light is λ = 520 nm.

1.2.3 Compute the ideal camera pixel size (Nyquist) and compare it to the actual pixel of the camera. Will the resolution be limited by diffraction or by the sampling of the camera?

3- If acquisition in TIRF

Robert is tempted by the possibility of making his acquisitions in TIRF in order to decrease the background signal and thus increase the contrast of his images. However, he wonders if the evanescent wave will allow him to excite the nucleus of the cells knowing that, at best, they are a few microns from the surface of the microscope slide.

1.3.1 What makes TIRF decrease the background signal?

1.3.2 What is the maximum angle of incidence (objective Numerical Aperture NA=1.45), in degrees, of the excitation beam that its microscope objective can excite the sample?

1.3.3 If Robert chooses an angle of incidence of 65° with a laser beam at λ_exc = 488 nm and his immersion medium is water (refractive index of water = 1.33), what is the depth of excitation of the evanescent wave?

4- If acquisition is confocal

Still with the idea of decreasing the background fluorescence, Robert is now tempted by the confocal mode since it would allow to reject the signal outside the focal plane thanks to the use of a pinhole.

1.4.1 What will now be the factor limiting the size of the field of view? Will Robert be able to observe his tissues in their entirety?

1.4.2 What will be the size of the scan step he will need to use to sample the image without sacrificing optical resolution?

1.4.3 Since the integration time per pixel is 10 µs, how long will it take to take an image?

1.4.4 The microscope has several pinholes to filter the signal outside the focal plane. Calculate the size of the pinhole that Robert should use.

Conclusion

Assuming that the contrast would have been sufficient regardless of the illumination mode, what mode of illumination of the sample would you recommend to Robert? Justify.

The sample would have been alive and the fluorescent markers would have been moving on time scales of the order of a second, which illumination mode would you then have recommended? With what compromise(s)?

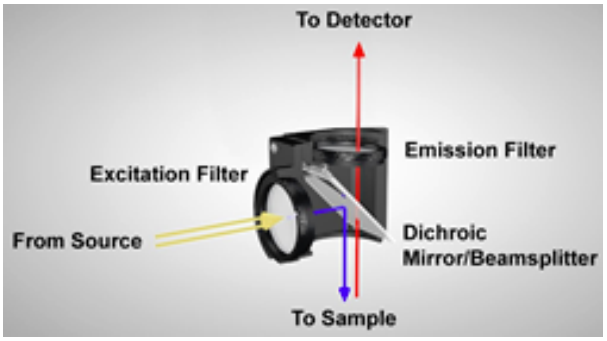
Other ressources:

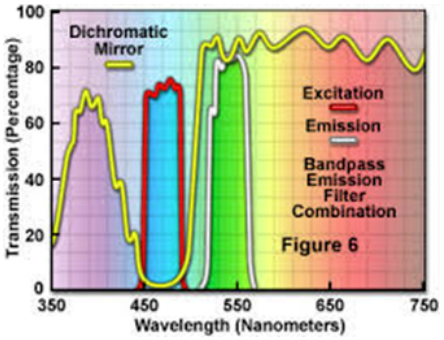
- Numerical aperture simulator
- Immersion oil simulator
- Objective focal lens simulator
- Microscope conjugated field planes
- Lens
- Tube lens and pupil
- Magnification

Part 2: Choosing the right filter set, excitation sources and fluorophores for LIVE cell sample.

1- Acquisition in Single Color

Simone would like to image live samples to visualize cell division during 1 hr. To capture the cell division events he is interested in, Simone needs bright and stable fluorescent proteins to label her sample knowing that her microscope contains 4 different filters cube sets:





DAPI cube	**GFP cube**
Excitation: Chroma AT350/50x	Excitation: Chroma AT470/40x

Dichroic: Chroma T400lp Emission: Chroma ET460/50m	Dichroic: Chroma AT495lp Emission: Chroma ET525/50m
RFP cube	**Cy7 cube**
Excitation: Chroma AT540/25x Dichroic: Chroma AT565DC Emission: Chroma AT605/55m	Excitation: Chroma ET710/75x Dichroic: Chroma T760lpxr Emission: Chroma ET810/90m

Simone has access to the Fluorescent Protein database (https://www.fpbase.org/) and all the tools on this Website (Spectra viewer) to choose the best Fluorescent Protein and filter sets (Dichroic mirror, excitation and emission filters) for Simone's microscope to do her experiment.

Using the same tools as Simone:

2.1.1 Plot the different cube spectra (Spectra viewer)

2.1.2 Which fluorescent protein would you recommend her to use? Overlay selected FP spectra with filters spectra.

2.1.3 Compare the following excitation source spectra : Mercury Lamp, LED and a laser. Overlay spectra. Justify your excitation source choice.

2.1.4 According to your settings (excitation and emission filters, objective transmission (objective reference: CFI Plan Apochromat Lambda 100X Oil) and collection efficiency (% of light collected by objective = -1/2 (cos (θ/2) -1), θ being the collection angle calculated in part 1) and Quantum Yield of your detector, calculate the collection efficacy of Simone's microscope configuration for this experiment.

For all these questions, please justify, comment and illustrate your answers.

2- Acquisition in Dual Colors

Simone now decided to look simultaneously at 2 different proteins. She wants to know if protein A and B co-localize in the Hela Cell nucleus. For this experiment, she decided to label protein A with eGFP and protein B with mCherry.

2.2.1 Does Simone have the right filters to perform this experiment?

2.1.2 What will be the best solution for Simone's experiment? Which filter cube would you recommend for this experience?

Justify, comment and illustrate your answers.

Part 3 : Atomic force microscopy

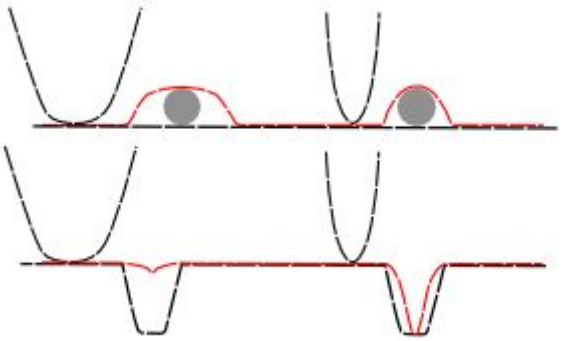
https://parksystems.com/medias/nano-academy/how-afm-works

-1- Properly evaluate the cantilever stiffness:

A silicon cantilever has a length L of 100 μm, a width of 10 μm and a thickness of 1 μm. The Young Modulus E of Silicon is nearly 130 GPa. Evaluate the cantilever stiffness k (with units) given by (see slides):

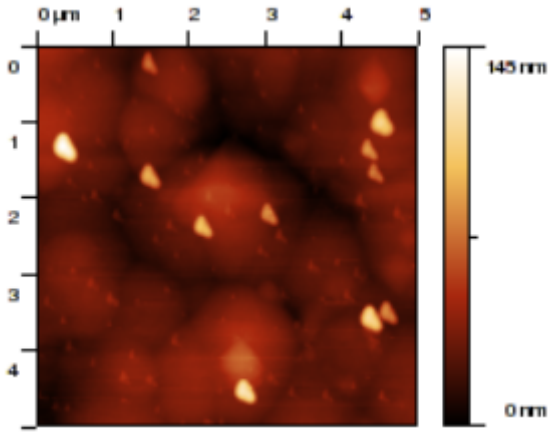
-2- Properly evaluate the lateral resolution:

As shown in the final slides of the AFM presentation, the lateral resolution that you can achieve depends on several parameters. At first, the AFM tip radius plays a major role since the measured morphology is affected by the convolution between the tip and the measured object shapes:

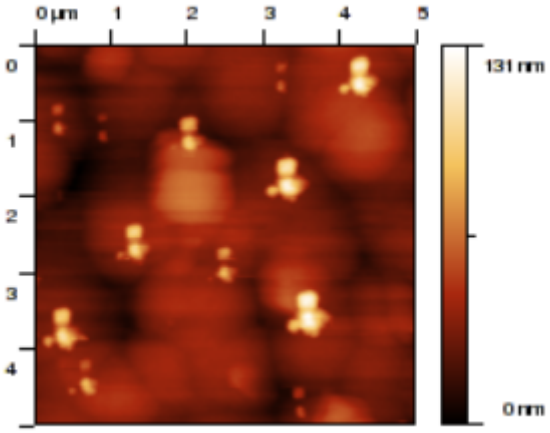


Credits: https://afmhelp.com

-2a- Can you draw the shape of the AFM tip that has been used to obtain the following image, where clearly the tip is larger than the measured objects?



And the following image?



-2b- Assuming the tip to be a non-contaminated sphere of 1 nm tip radius, if it is used to image isolated spheres of 10 nm tip radius, what is the diameter of the spheres in the AFM image (lateral resolution)? What is the measured height of the spheres (vertical resolution)?

-3- Properly evaluate the acquisition time of an AFM image:

We run an experiment where a force versus distance curve is acquired in each pixel of the AFM image (i.e. using the QI mode). The image is recorded with 256 lines with 256 pixels. Assuming that the speed of the cantilever is 100 µm/s and that each force curve is 1 µm long (1 µm for the approach and 1 µm for the retract), what is the acquisition time of each pixel? And what is the approx. total acquisition time of the AFM image?

-4- Properly evaluate the resonance frequency and oscillation amplitude of an AFM cantilever:

The cantilever can be modelled as an harmonic oscillator (see slides)

$$m\ddot{z} + \delta\dot{z} + kz = F_{interaction}(z) + F_0(\omega t)$$

Assuming no interaction force between tip and sample (cantilever far from the sample)

$$F_{interaction}(z) = 0$$

And the equation can be easily solved, leading to the oscillation amplitude

$$A = |z| = \frac{\frac{F_0}{m}}{\sqrt{(\omega_R{}^2 - \omega^2)^2 + \omega^2\delta^2}}$$

Let's assume the cantilever is in vacuum (δ =0), what is the oscillation amplitude at resonance (ω = ω_R)?

Now let's assume the cantilever is immersed in water, the Q factor of the resonance in water is given by

$$Q = \frac{\omega_R}{\Delta\omega}$$

Where Δω is the full width at half maximum of the resonance.
The Q factor and the damping factor δ are related by

$$Q = m\,\omega_R / \delta$$

Assuming the cantilever to be a sphere of approx. 10 µm radius, in water $\delta = 10^{-8}$ N s / m.
The cantilever Q factor is equal to 2000 in water.
Evaluate the Q factor if the cantilever was immersed in honey where $\delta = 2\ 10^{-5}$ N s / m.

Try to draw the corresponding resonance frequencies (Amplitude as a function of the frequency) in water and in honey.