# Assessing Nuclear Pore Complex (NPC) density using fluorescence microscopy and image analysis

## **Objectives of the practical**:

The purpose of this practical is to use fluorescence microscopy to understand the role of specific nucleoporins in nuclear pore complex assembly. The main objectives are:

I. Understand the problematic : to decipher the mechanisms of NPC assembly, how can you label, image, identify and count mature nuclear pores (=NPCs) *vs* pores being assembled (=PIs, for pore intermediates) - you will find inspiration in Talamas et al, 2011. In this practical, you will have several samples of cells, either mock-transfected, or transfected with an siRNA against Nup133.
II. design an immuno-labeling protocol in order to differentially label NPCs and PIs .
III. design an imaging protocol in order to compare the following imaging modalities :
    - epifluorescence
    - confocal
    - airy-scan
IV. develop an image analysis framework to measure the density of NP w/wo siRNA.
V. conclude on the suitability of each technique to accurately measure NPC density.

At the end of this practical, you should be able to :

- explain the principles of the microscopy techniques you used
- justify the experimental choices you have made for NPC imaging
- detail your analysis protocol

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# Resources :

All resources are available at this location:
https://drive.google.com/drive/folders/1Ctzl8R3-WHGS1UtkUafXr65HmXByWaFE?usp=share_link

Resources regarding Nuclear Pore Complexes (biology and imaging):

- Talamas et al, 2011

Resources regarding confocal microscopy :

- Jonkman et al. - 2020, Nat. Protocoles.
- https://www.leica-microsystems.com/science-lab/pinhole-effect-in-confocal-microscopes

Resources regarding image deconvolution :

- https://svi.nl/Huygens-Deconvolution - Huygens is used by many biologists / microscopists to perform deconvolution on fluorescence images. This software will be accessible during this practical
- https://www.olympus-lifescience.com/fr/microscope-resource/primer/digitalimaging/deconvolution/deconintro/ - An interesting resource explaining what are the sources of image degradation and how deconvolution could be used.

Resources concerning airy-scan microscopy :

- Huff - 2015, Nat. Methods : https://www.nature.com/articles/nmeth.f.388
- https://www.zeiss.com/microscopy/int/products/confocal-microscopes/compact-confocal-lsm-900-with-airyscan2-for-multiplex-fluorescence-imaging.html?vaURL=www.zeiss.com/microscopy/int/products/confocal-microscopes/lsm-800-with-airyscan.html - see the technical note on the basic principle of airy-scan at the bottom of the page

This statement will guide you through all the steps of the practical. It will also be useful for you to gather all the relevant information to write your report (including introduction, material and methods, results, conclusions….)

# I. Problematic

Reading Talamas et al will help you understand the problematic and the approaches you will use to decipher key players involved in NPC assembly.

- ☐ When do NPCs assemble ?
- ☐ Why is it a complex and poorly understood mechanism ?
- ☐ How can we differentiate nascent vs mature pores by fluorescence ?
- ☐ What approach can be used to identify key players in pore assembly ?

# II. Sample preparation :

In these practicals, you will image Nuclear Pore Complexes (NPCs) directly in cells.
Two different samples will be prepared beforehand: control cells, and cells in which Nup133 (a component of NPCs) has been depleted.

- ☐ What signal do you expect from your microscopy images ?
- ☐ What difference do you expect between the two conditions ?
- ☐ How can you quantify this ?
- ☐ Do you know a way to differentiate fully assembled NPCs and pore intermediates ?

Before the practicals, we will seed cells on glass coverslips and transfect them with siRNAs:

- scrambled (control condition)
- against Nup133

Cells will be fixed and ready to label.
So you will get a 6-well plate: 2 wells contain a 25mm coverslip with cells transfected with scrambled siRNA (labeled “sc”) or siRNA against Nup133 (“si133).

1- Here is a list of reagents and antibodies available for sample preparation. Based on this list, and helped by the publication (Talamas et al, 2011).

- ☐ Choose primary antibodies to label mature pores and pore intermediates.
- ☐ Choose appropriate secondary antibodies (also check the configuration of the microscope) - what can you tell about respective resolutions ?
- ☐ Ideally, what should you do to check that the labeling is specific ?

Immuno-fluorescence buffer (PBS + 1mg/ml BSA, 0.1% Triton, 0.02% SDS)
PBS
Hoechst (1mg/mL)

Primary antibodies:

| Supplier | Ref | Protein | Type | Immunogen | Notes |
|---|---|---|---|---|---|
| abcam | ab124770 | **SUN1** | rabbit mono [EPR6554] | Synthetic peptide, aa 100-200 of hSUN1 | IF (1:1000) |
| Covance | | **mAb414** | mouse mono | | 1:1000 |
| Santa Cruz | sc376763 | **Nup133** | mouse | | Kane et al, 2018, works OK for STED (1/200) |
| Abcam | ab64276 | **RanBP2** | rabbit | | Kane et al, 2018 |
| Abcam | ab84516 | **Tpr** | rabbit | Synthetic peptide, C -ter, AA 2300-2349 of hTPR | McCloskey et al, 2018 (1:500) |
| Genetex | GTX102128 | **POM121** | rabbit | | McCloskey et al, 2018 (1:500) |
| abcam | ab53540 | **ELYS** | mouse | | Kane et al, 2018 |
| abcam | ab96462 | **Nup153** | mouse [SA1] | | McCloskey et al, 2018 (1:500) |

Secondary antibodies:

| | Dye | Dilution |
|---|---|---|
| anti-Mouse | AlexaFluor 488 | 1:1000 |
| anti-Mouse | AlexaFluor 594 | 1:1000 |
| anti-Mouse | AlexaFluor 647 | 1:1000 |

| | Dye | dilution |
|---|---|---|
| anti-Rabbit | OG 488 | 1:1000 |
| anti-Rabbit | AlexaFluor 568 | 1:1000 |

| | | |
|---|---|---|
| anti-Rabbit | AlexaFluor 647 | 1:1000 |

2- Design the detailed protocol to label the cells by immuno-fluorescence.
All labelings will be done directly in the 6-well plate. Count 0.5mL of solution per well.
Antibodies are very expensive (~ 400€ / 100uL) so always try to minimize the quantity you use.

- ☐ list of IF mixtures to prepare beforehand
- ☐ protocol (incubation times and washes)

# II. Description of the microscope :

For this practical, you will work on a LSM800 microscope, from the company Zeiss. This microscope is equipped with two imaging side-ports :

1. the left port is used for confocal and airyscan microscopies
2. the right port is equipped  for epifluorescence.

In the following sections, the main optical components used for each imaging modality are described. A few questions are added to test your theoretical knowledge and help understand what are the specificities of each technique.

## 1. Epifluorescence :

A side view of the microscope right side is presented below :

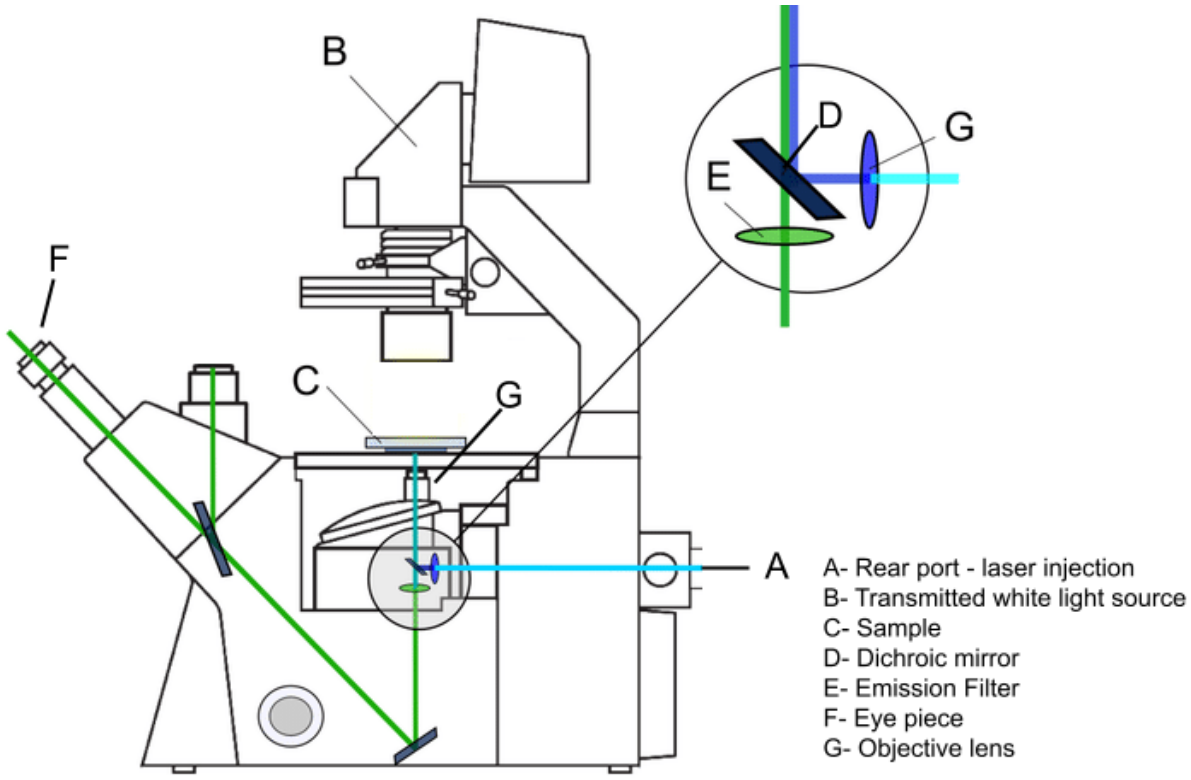



*Fig 1 : Side view of the microscope*

Briefly, the microscope is composed of :

- a fibered laser source, plugged into the rear port of the microscope (A). The following wavelengths are accessible : 405nm, 460nm, 477nm, 520nm, 546nm, 638nm and 750nm.
- a dichroic mirror (D, see spectrum on Fig 2)
- an emission filter (E, see spectrum on Fig 3)
- a 63x water immersion objective with a numerical aperture NA=1.2 (Zeiss, G)

- a sCMOS camera (Hamamatsu, Orca Flash 4 - v3). The detector of the camera is composed of 2048x2048 pixels with a physical size of 6.5 x 6.5µm² each. The spectral properties of the camera are displayed on Fig 4

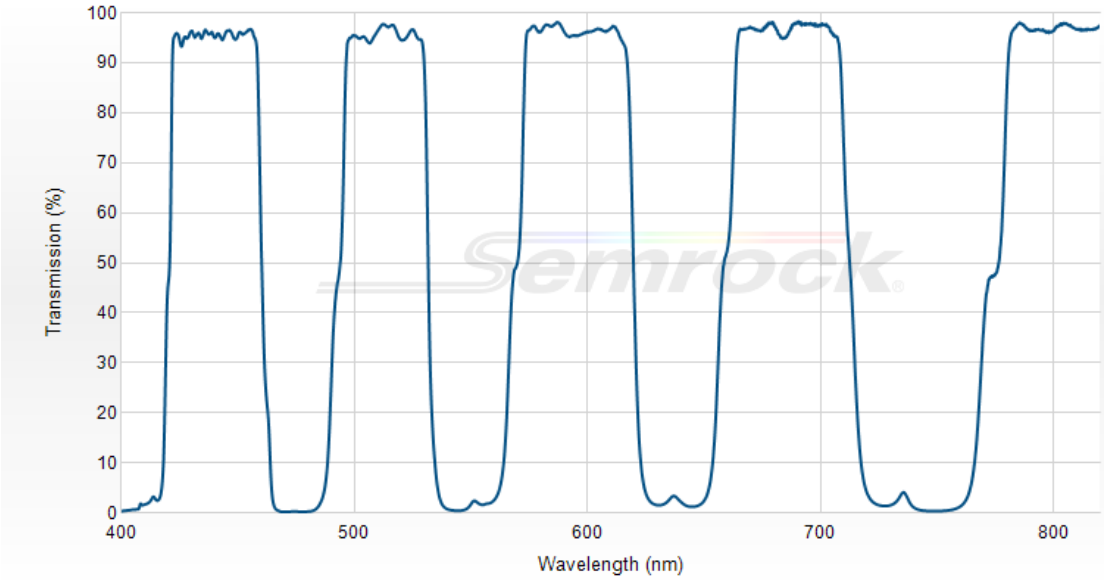



*Fig 2 : Spectrum of the dichroic mirror used for epifluorescence on the microscope*

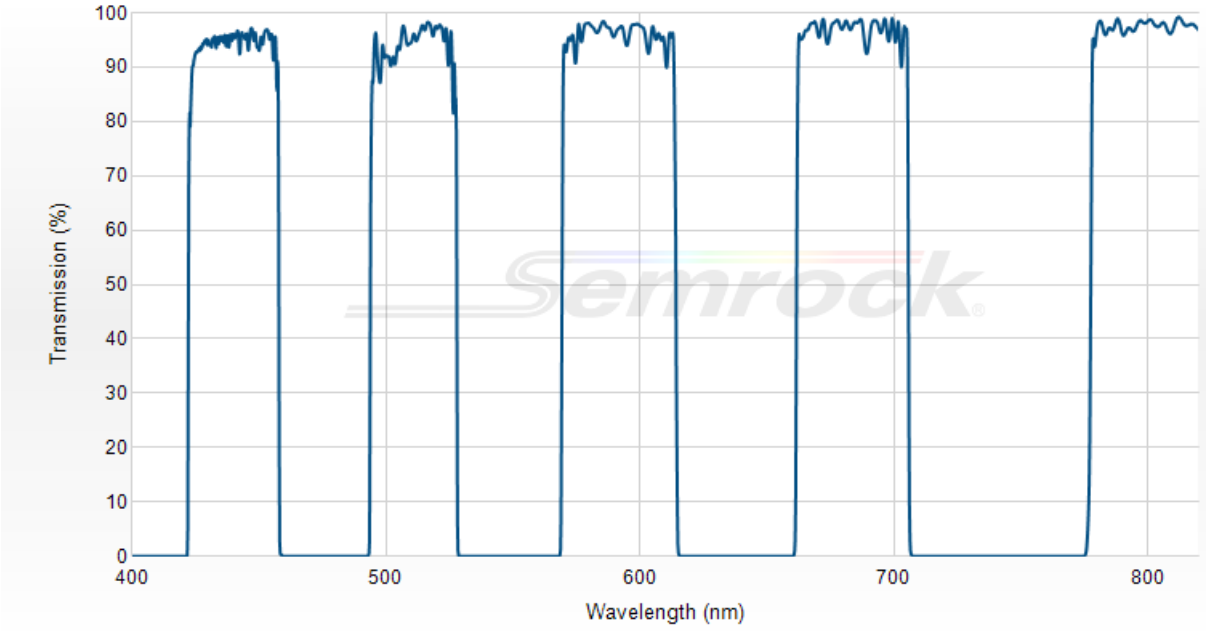



*Fig 3 : Spectrum of the emission filter used for epifluorescence on the microscope*

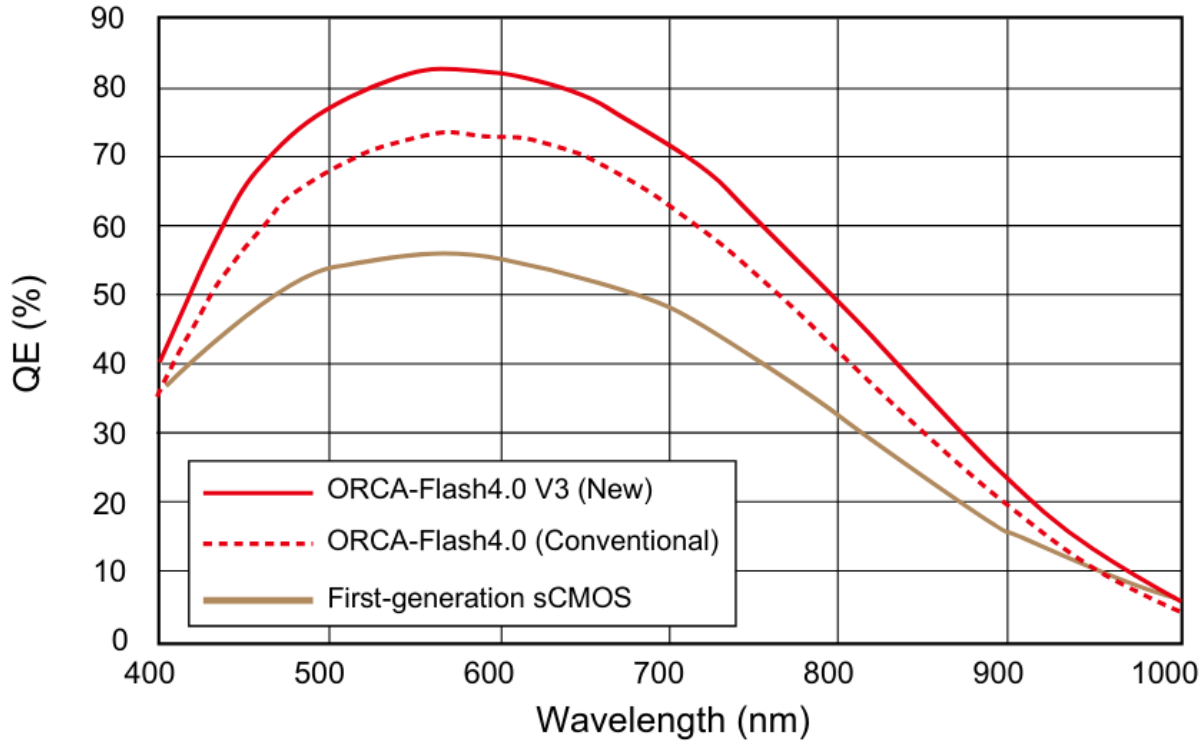
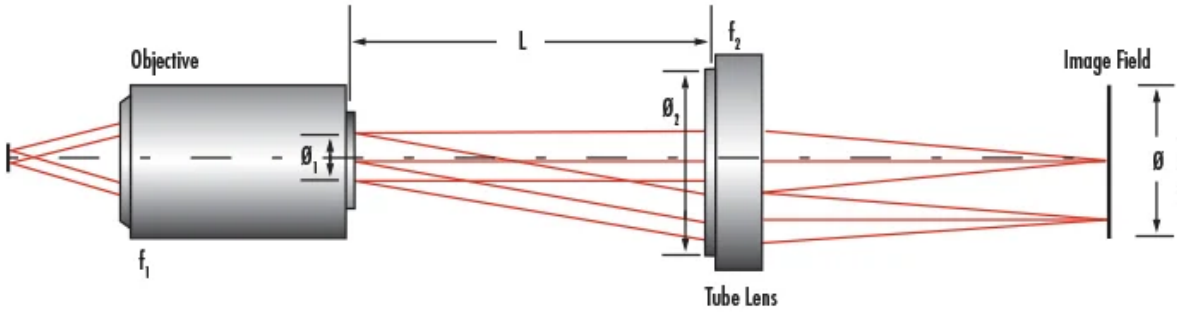



*Fig 4 : Spectral response of the sCMOS camera*

## Preliminary questions :

- ☐ According to the spectra of the dichroic mirror and the emission filter, which laser lines are accessible on the microscope?
- ☐ Considering the excitation and emission spectra of the dyes used for labeling NPC, is the microscope optimum for imaging these fluorophores? Why?
- ☐ What is the effective pixel size in the object plane? Comment.
- ☐ What is the size of the field of view images by this microscope?



- [ ] As illustrated above, the microscope imaging system is composed of an objective lens (f1) and a tube lens (f2). The tube lens has a diameter of 1”=25.4mm, a focal length f2=164.5mm and is separated from the objective lens by L=121.1mm. Calculate the maximum size of the observation field (Field of View) ⌀ without vignetting :

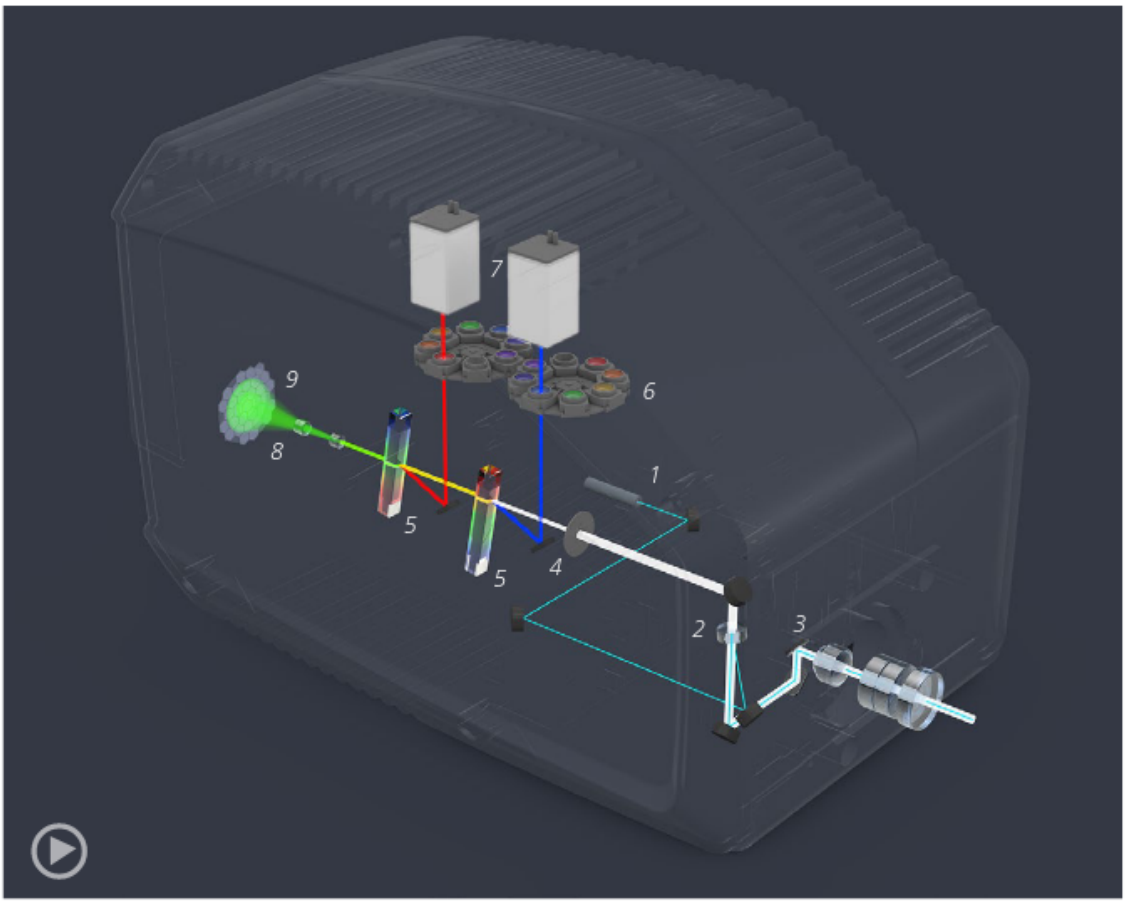
$$L = \left( \frac{\emptyset_2 - \emptyset_1}{\emptyset} \right) f_2$$

- [ ] Using the description of the microscope, calculate the PSF for each fluorescent dye you will use. How does it compare to the typical size of a nuclear pore ?

## 2. Confocal microscopy :

A simplified design of the confocal head is represented on Fig. 5. Briefly, the microscope is composed of :

- four **laser lines** : 405nm, 488nm, 561nm and 640nm.
- **two galvo mirrors** allowing the XY-scanning of the sample by the laser
- a **water immersion objective**, with a NA=1.2 and a magnification of 63x
- a **mechanical pinhole** whom aperture is fully controlled by the microscope software
- three **confocal GaAsP detectors (see Fig.6 for their detection properties)** :
    a. two of them are regular GaAsP detectors and are associated to the detection channels #1 and #3
    b. a third one is the airy-scan detector, associated to the detection channel #2




***Schematic beam path of LSM 800***

*1. Excitation laser lines*
*2. Main beam splitter (MBS)*
*3. Galvo scanning mirrors*
*4. Pinhole*
*5. Variable Secondary Dichroic (VSD)*
*6. Emission filters*
*7. Confocal detectors*
*8. Zoom optics*
*9. Airyscan detector*

*Fig. 5: Scheme of the confocal detection head of the LSM800 microscope*

As illustrated by the microscope scheme, each detection channel is optimize for a specific range of the visible spectrum :

- detection channel 1 for the blue/UV part of the spectrum
- detection channel 2 for the yellow/green wavelengths
- detection channel 3 for the red/NIR

in order to adjust the detection wavelengths of each channel, two **variable dichroics VSD** and two **filters wheels** are accessible for the detection channels #1 and #3 respectively.

The microscope is fully controlled by a software called ZEN-blue.

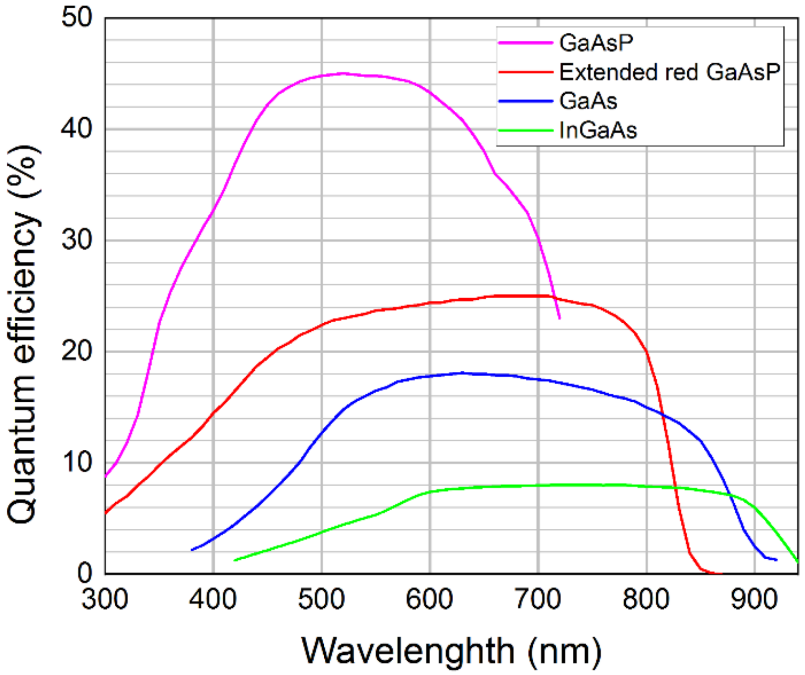



*Fig. 6 : Quantum efficiency of typical single-point detectors.*

## Preliminary questions :

- ☐ Comment on the properties of the GaAsP detectors as compared to the sCMOS camera used for epifluorescence microscopy

- ☐ For the same dye and with the same excitation condition, a confocal microscope allows a slightly better resolution than an epifluorescence microscope. Why?

- ☐ According to the excitation/emission spectrum for DAPI and Alexa Fluor 568 (see Fig. 7), which laser line should be used to image each fluorophore? Which detection channel?

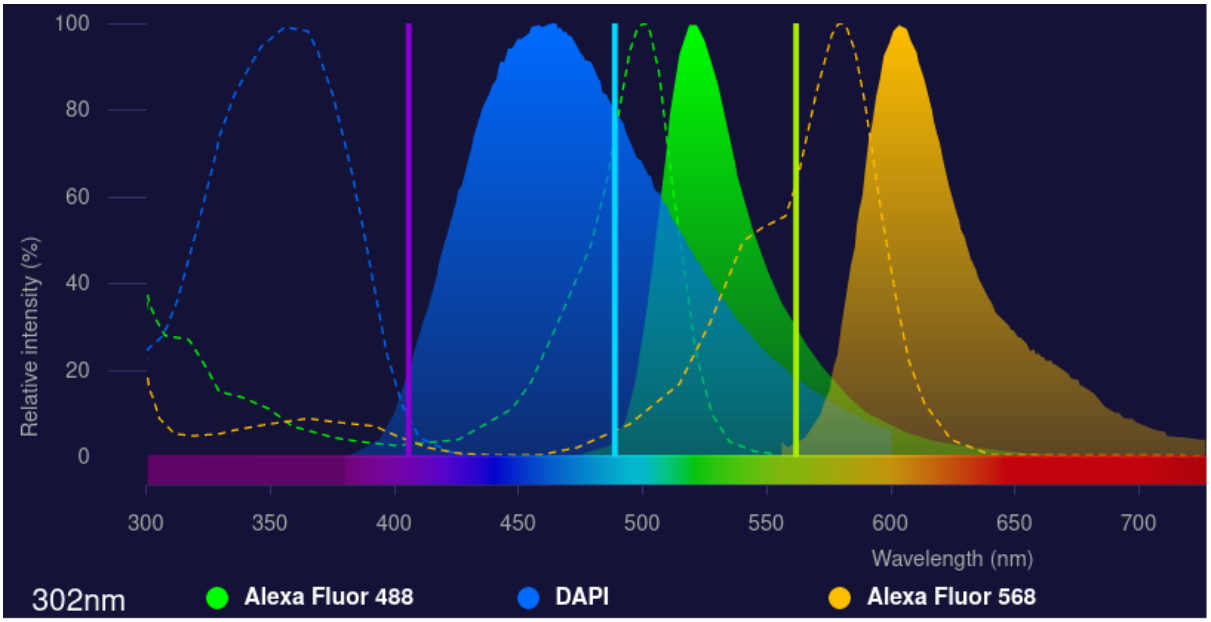



*Fig. 7 : Excitation (dotted line) and emission spectrum of DAPI (blue), Alexa Fluor 588 (green) and Alexa Fluor 568 (yellow).*

- ☐ Using the description of the microscope, calculate the excitation PSF for each fluorescent dye. How does it compare to the typical size of a nuclear pore ?

- ☐ According to these values, what would be the optimal pixel size for imaging each dye?

- ☐ The pinhole diameter is expressed in Airy unit (AU), that is the diameter of the central peak in an Airy pattern. Why is it more interesting to use this unit rather than actual distance? What would be the size (in µm) of a 1AU pinhole aperture for each fluorescent dye.

# 3. Airy-scan microscopy :

The microscope LSM800 offers the possibility to work with an Airy-scan detector, enabling an increase in resolution by ~1.7 fold in all directions.

Both confocal and airy-scan imaging are laser-scanning techniques. However, as illustrated on Fig. 8, the two techniques rely on very different imaging path and detectors :

- For the confocal, a single GaAsP detector is used to monitor the fluorescence signal emitted from the diffraction-limited excitation volume. A pinhole is used to remove out-of-focus fluorescence, leading to an increase in optical sectioning and sometimes in resolution.
- For the airyscan, a specific detector is used (see Fig. 8). It is composed of 32 single GaAsP detectors, assembled in a honey-comb like pattern. The optical path is modified in such a way that the signal coming from the excitation volume is now distributed onto the whole detector. As illustrated on Fig. 8 & Fig. 9, the central part of the diffraction pattern is then fully imaged by the airy-scan detector and each single detector is acting as a pinhole with an aperture of ~0.2AU.

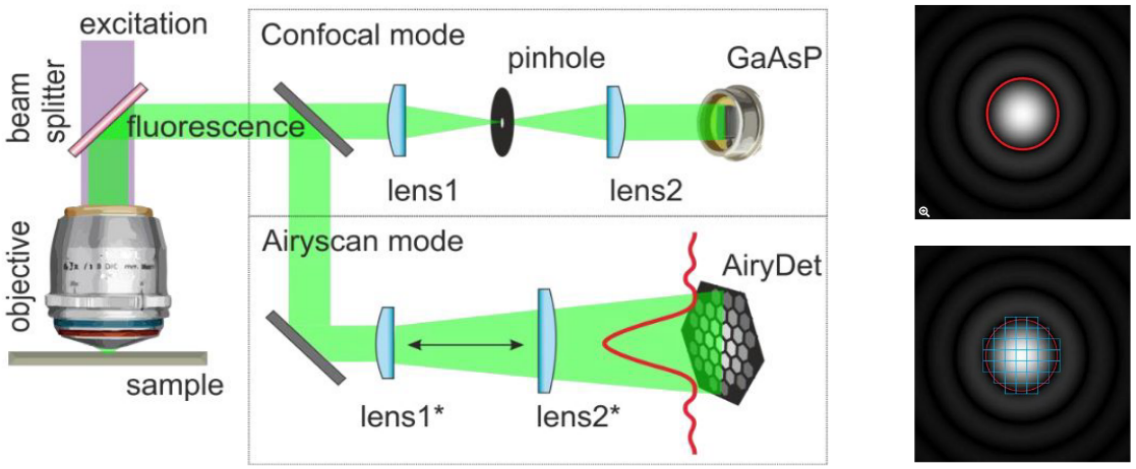



*Fig. 8 : Comparison between confocal and airy-scan imaging*

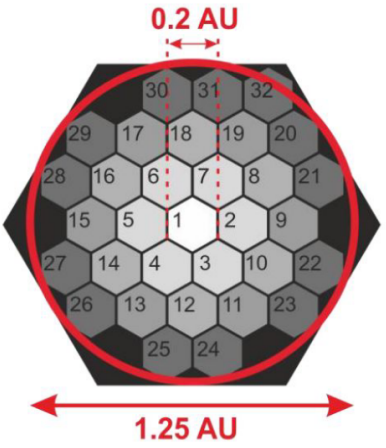



*Fig. 9 : Airyscan detector. The detector is actually composed of 32 individual GaAsP detectors, assembled in a honey-comb pattern.*

In confocal microscopy, closing the pinhole below 1AU would lead to an improvement in XY resolution by a factor of ~1.4 (see Fig. 10) and up to 2 after deconvolution. In practice however, closing the pinhole to 0.2AU would mean losing >95% of the fluorescence light. Therefore, only very bright and stable samples could be imaged in these conditions.

The idea behind the airyscan detector is to allow confocal imaging with a 0.2AU pinhole without losing signal. Since each detector is acting as a single pinhole, all the fluorescence light is collected and mathematically “reassigned” using a specific deconvolution algorithm. This imaging technique is allowing an improvement in resolution by 1.7 after deconvolution.

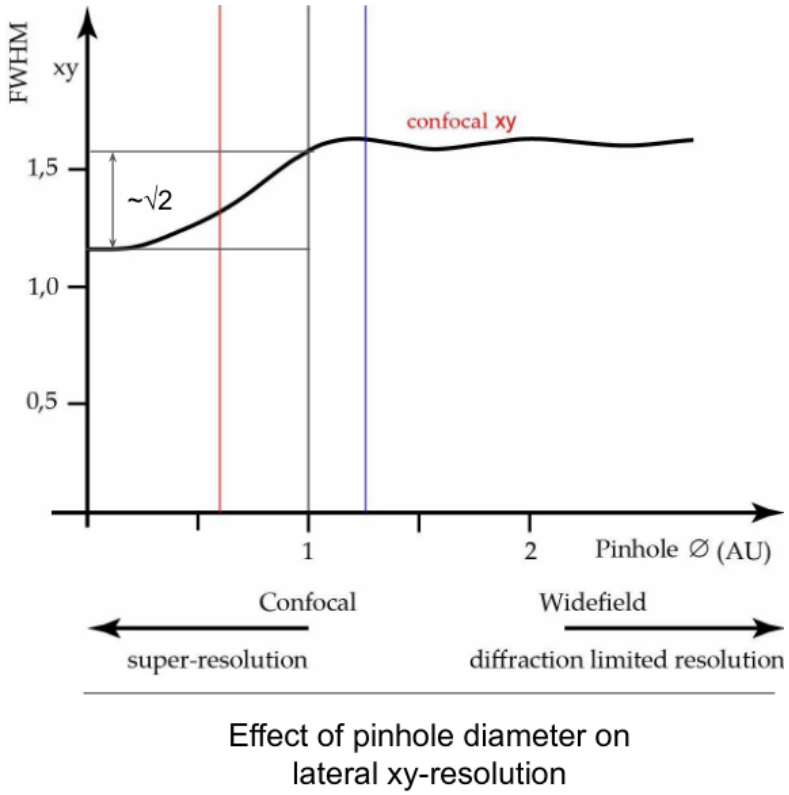



*Fig. 10 : Effect of the pinhole aperture on the lateral resolution in confocal imaging*

# IV. NPC imaging and effect of siRNA treatment :

The aim of this section is to design an experimental protocol to **measure the variation in NPC density depending on the siRNA treatment.** To do so, you will need :

- ☐ To image NPC with all imaging modalities
- ☐ Estimate the size of NPC for each technique and compare it to the theory
- ☐ Is deconvolution useful to improve NPC detection?
- ☐ Justify which technique(s) is best suited to answer your biological question (discuss the pros and cons - produce images to illustrate / support your statements)
- ☐ Develop and optimize your imaging protocol to evaluate the density of NPC depending on the sample.
- ☐ Acquire enough data for your analysis and obtain statistically relevant results
- ☐ Develop your own analysis pipeline in Python in order to quantify NPC density for each condition (see section V.)

# V. Image analysis

You will have to write a script in Python to

- detect pores (NPCs and PIs)
- calculate their density / the total number of pores per nucleus in cells treated with scrambled and siRNA.

- ☐ Think of the parameters you will need to extract from your images
- ☐ Think of the functions / methods you could use to achieve this
- ☐ Do not forget preliminary image treatments that may be required (denoising, normalization…)
- ☐ Once you have extracted the parameters of interest, how will you plot these ?
- ☐ Statistics

From these guidelines, build an algorithm that we will discuss together before coding *per se*.