Important equations for light microscopy

1. Background knowledge

1.1. Definition of resolution

The resolution is a distance. Its unit in light microscopy is usually in nm. The resolution of an objective is the smallest distance between 2 points in the sample that the objective can resolve (i.e. we can tell with certainty that there are 2 dots. They do not appear fused as a single dot). When the resolution value decreases, we can resolve dots that are closer to each other, so the resolution improves, i.e. 100 nm resolution is better than 200 nm resolution.

There are 2 types of resolution to consider: the xy and z resolutions delivered by the objective (optical resolution) and the xy and z resolutions at which we record the images (digital resolution).

1.2. Choice of equations in this course

If you search online how to calculate the xy and z resolutions for light microscopy, you will find several equations, mostly related to the optical resolution. Each of these equations is only valid:

- in a specific context (for example, imaging with a very closed confocal pinhole),
- if the digital resolution fulfils the Nyquist sampling theorem,
- last but not least, if the sample is perfect. In biology, sample imperfection often limits the resolution in images.

Unfortunately, it is common for websites to mention an equation without specifying any of these 3 points. This leads to a lot of confusion.

In this course, **for the sake of simplicity**, we will use the same xy and z equations for the optical resolution, regardless of the imaging modality (confocal or widefield) and context (pinhole closed or open). In the course, we also put a lot of emphasis how to prepare high-quality samples.

For the same reason, we will also use for Nyquist sampling the commonly used factor of 2.3, although anything from 2 to 4 can also be valid, again depending on the context.

1.3. In all equations below:

- n is the refractive index of the objective immersion medium
- λem is the wavelength of the emitted light
- NA is the numerical aperture of the objective
- Mag is the total magnification (magnification of the objective * extra magnification)

1.4. Refractive indices (n)

Refractive index (n)	1	1.33	1.47	1.52
Medium	Air	Water	Glycerol/silicon oil	Oil
Sample type	N/A	Cell culture	Most uncleared tissues	Cleared tissues

2. Brightness of the objective

NA^4/Mag^2

3. Optical resolution (resolution of the objective)

The optical resolution is the resolution delivered by the objective at a given wavelength and is limited by diffraction **if the sample is perfect**.

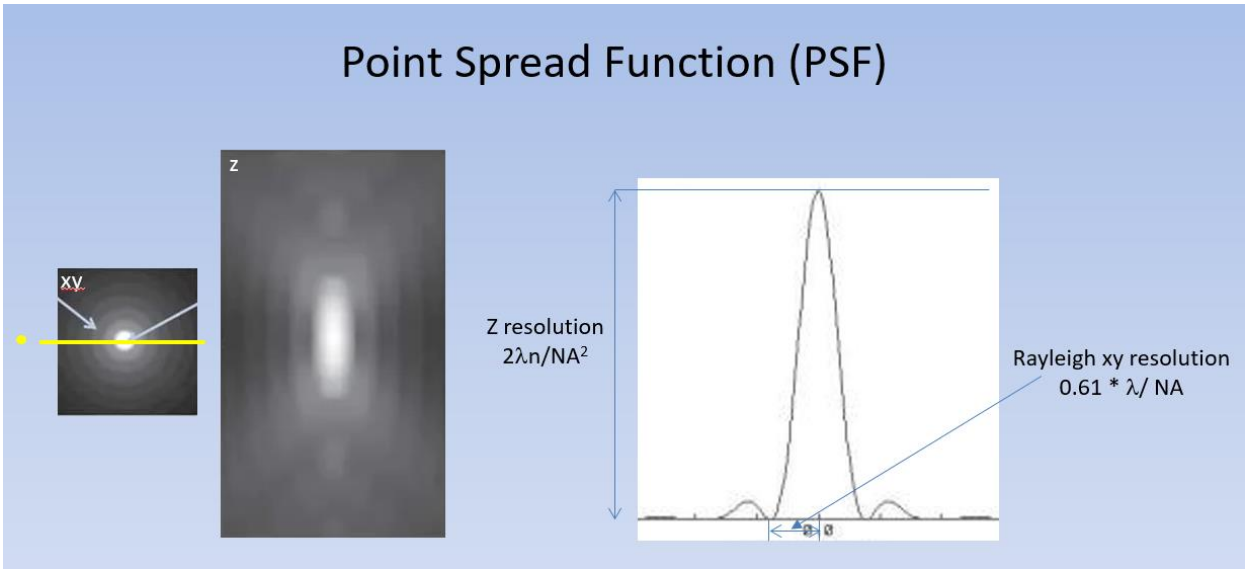
3.1. xy optical resolution (= xy radius of the PSF main lobe = Rayleigh equation)

$0.61*\lambda em/NA$

3.2. z optical resolution (= z radius of the PSF main lobe = objective depth of field).

More details here.

$2n*\lambda em/NA^2$



4. Nyquist sampling theorem (= digital resolution = the way we record images)

Let's imagine that we want to watch a high-resolution movie at home. The resolution of the TV used to play the movie must match or exceed the resolution of the movie, if we want the movie to be displayed at its best quality. If the TV is not good enough, we will not be able to view the movie at its best quality.

The same is true in microscopy. If we record the image produced by the objective with large pixels (low digital resolution), the final image will not display the fine details resolved by the objective.

The Nyquist sampling theorem tells us how small the image pixels should be for the image to fully display the optical resolution.

4.1. Required xy image pixel size (in nm) to reliably record all details resolved by the objective in the xy direction

According to the Nyquist theorem, the pixels in the image must be at least 2.3 time smaller than the xy optical resolution.

$$(0.61*\lambda em/NA)/2.3 \text{ (or smaller)}$$

The size of the image pixels is always indicated somewhere in the microscope software if the microscope is calibrated (which is the case for all automated microscopes). The image pixel size is also recorded in the image metadata, if the image was saved in a way that preserves metadata, in general the format recommended by the microscope manufacturer.

Imaging with pixels that are larger than the number given by this equation is called xy undersampling.

On single-point confocals, one can freely choose the xy pixel size by:

- choosing more or less pixels in a given scan area, or
- zooming more or less, for a given number of pixels.

A convenient button (usually called Nyquist or Optimal) is found in the software of single-point confocals to adjust the xy pixel size to the Nyquist theorem.

On camera-based systems, there is no such button. Cameras have recording devices that are confusingly called camera pixels. Their size is in um (found in the camera specifications). The resulting pixel size in the image is fixed for a given magnification.

On some microscopes, an extra magnification lens can be added, allowing you to adjust the image pixel size without changing the objective.

$$\text{Image pixel size} = \text{Camera pixel size}/\text{Mag}$$

4.2. Required z step (in nm) to reliably record all details resolved in the z direction by the objective

According to the Nyquist theorem, the z step in a z stack must be at least 2.3 time smaller than the z optical resolution.

$$(2n*\lambda/NA^2)/2.3 \text{ (or smaller)}$$

On any type of microscope, one can freely choose the size of the z step to acquire a z stack. A convenient button (usually called Nyquist or Optimal) is found in the software to adjust the z step to the Nyquist theorem.

Acquiring a z stack with a z step larger than the number given by this equation is called z undersampling.

5. What is the best resolution that a camera can resolve?

Because the optical image (PSF) is magnified before being projected on the camera pixels, magnification matters for cameras.

Image pixel size *2.3 = (Camera pixel size/Mag) *2.3

6. What happens when we undersample?

We first estimate the smallest distance (in xy and in z) that we need to resolve in the images to reliably answer our scientific question. We then need to check the following:

- The sample quality must be good.
- The objective must be able to resolve this required distance.
- The way we record each image must produce pixels that are 2.3 times smaller than the xy distance resolved by the objective, or even smaller.
- Z stacks must be recorded with a z step of 2.3 times smaller than the z distance to be resolved, or even smaller.

If all points above are fulfilled, the image resolution is the same as the optical resolution.

If the last 2 points are not fulfilled, we undersample. When this happens, the resolution in the image (or the z stack) is limited by the way we record (digital resolution/sampling) instead of by the optical resolution. When we undersample, the resolution in the image becomes 2.3x the image pixel size (or the z step in a stack) instead of being what the objective resolves.

- **Actual xy resolution in an undersampled image:**

  Image pixel size *2.3

- **Actual z resolution in an undersampled image:**

  Z step *2.3

Additionally, all undersampled details create **aliasing artifacts** that not distinguishable from the real sample.

7. Sampling in time

The Nyquist theorem is conveniently applicable to measuring intensity variations over time but because time is analogue, not digital, the Nyquist factor is 2. One needs to consider the sample and scientific question to determine the **time necessary for a significant change to occur in the phenomenon one wants to record**. The time interval that fulfils the Nyquist sampling theorem is equal to half that time or smaller.

References:

Leica website about resolution

Tips:

Download the nice ‘Resolution’ app which calculates it all. 😊

Android: https://play.google.com/store/apps/details?id=com.Barlowax.resolutionfragments

iOS: https://itunes.apple.com/us/app/microscope-resolution/id1229786939?ls=1&mt=8