Fluorescence Microscopy : SMLM and STORM Imaging of Microscopic Biological Structures

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Introduction

Fluorescence microscopy appeared in the 20th century, where specific molecules were labelled with fluorescent markers to be localised. Due to the laws of diffraction, the maximum resolution that can be reached is of the order of the wavelength of the light of observation, meaning anything smaller than half the wavelength cannot be distinguished one from another. This made impossible to observe the cells in detail, so their structure at smaller scales were unknown. With some developments, few techniques emerged and this poster presents the use of the PSF in SMLM and STORM imaging. It shows how these techniques based on epifluorescence microscopy allow an accurate study of the following microscopic biological structures: mitochondria, clathrin and microtubules. [1]

Epifluorescence Microscope

The working principle of the fluorescence microscopy is to make samples light emitters by labelling the molecules of interest with fluorescent markers, called fluorophores. These will absorb excitation photons and emit by de-excitation photons that are detected and allows the localization of the molecule [2].

- **Infinity corrected microscope :** sample plane matches with front focal plane of the objective lens
  ⇒ image of the sample at infinity
  ⇒ needs tube lens to construct the image of the sample on the plane of the camera sensor
- **Infinity space :** this space between the two lenses has advantages : no magnification when its length changes and any optical element inside does not change the focal plane position nor adds spherical aberration
- **Epi-illumination :** both excitation and emitted lights go through same objective lens
  ⇒ epifluorescence

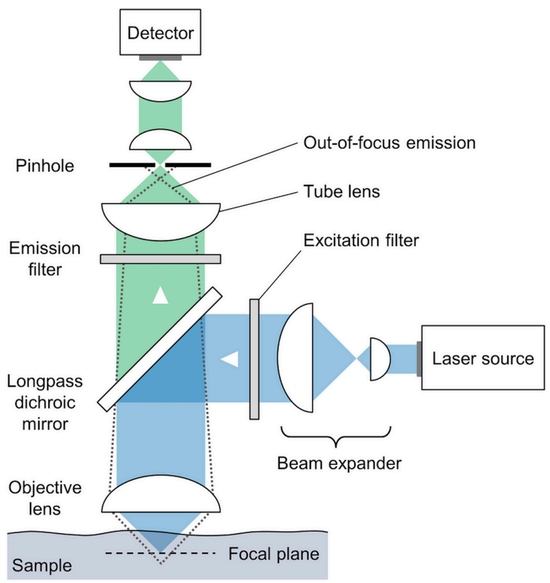

Fig. 1: Scheme of a modern infinity corrected epifluorescence microscope [3]

- Conversion of photonic signal into numerical signal in camera through photoelectric effect

SMLM and PSF

- **PSF (Point Spread Function) :** image of an infinitely small object emitting isotropic light that measures the quality of the imaging system
- **SMLM (Single Molecule Localization Microscopy) :** technique based on the PSF which gives localization of a single molecule with a precision of the order of 10 nm [1] by finding the center of the molecule with statistical approach

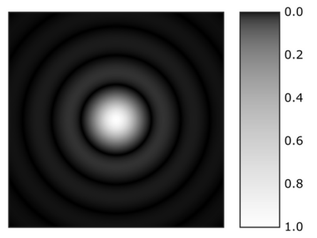

Fig. 2: Airy pattern [1]

The PSF is modeled by the Airy disk of maximum intensity I_0 and of intensity profile

$$I(X) = I_0 \left[\frac{2J_1(X)}{X}\right]^2 \tag{1}$$

with $X = 2\pi r NA/\lambda$, $J_1(X)$ the first-order Bessel function of first kind and NA the numerical aperture of the microscope. The radius of the Airy disk is $\Delta r \approx 0.61\lambda/NA$ [1]. The profiles obtained observing isolated polystyrene beads (emitters) with different wavelength are approximated by a Gaussian function

$$f(\mathbf{x}) = a \exp\left(-\frac{(\mathbf{x}-\mathbf{x_0})^2}{2w^2}\right) + b \tag{2}$$

with $\omega \approx \Delta r/3$ [1] the RMS width of the Gaussian and $\mathbf{x_0}$ the position of the center of the a bead.

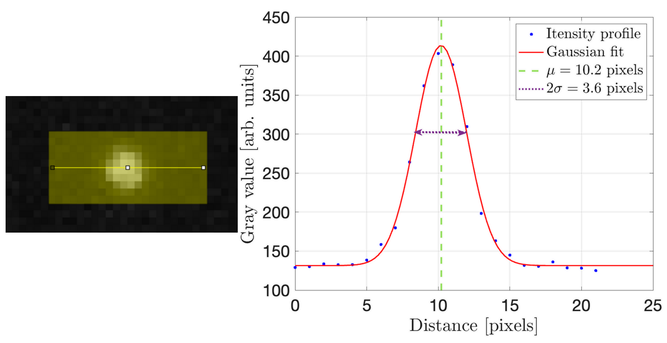

Fig. 3: Picture centred on a single bead with the region where the intensity profile plotted has been measured

	Red	Green	Blue	UV
λ [nm]	642	651	488	400
$\bar{w}$ [nm]	172 ± 3	206 ± 2	172 ± 5	197 ± 5
σ [nm]	4.5	4.4	3.7	3.8

Tab. 1: Mean PSF width $\bar{w}$ and localization precision σ obtained with different laser sources of wavelength λ

- **Intensity profile :** Airy disk intensity profile well approximated by a Gaussian curve in Fig. 3
- **PSF width :** characteristic PSF width about 250 nm [1]
- **Localization precision :** the order of magnitude of σ is of some nanometers
- No links with the wavelength highlighted
- **Error sources :** out-of-focus fluorescence, noises, aberrations, Airy disk model, ...

STORM Imaging

STORM (STochastic Optical Reconstruction Microscopy) imaging is a super-resolution single molecule imaging technique that permits to overcome the diffraction limit by smartly use the photoswitching mechanism.

- **Photoswitching :** stochastic switching of the fluorophores between ON and OFF states ⇒ random blinking creating on a given frame regions with isolated molecules ⇒ statistical localization with PSF can be applied in the reconstruction process
- **Fluorescence photophysics :** difference in rate allows distinguishing ON and OFF states
- **Procedure :** several frames of ROI (Region Of Interest) in different blinking state ⇒ SMLM on each frame ⇒ combination through batching process gives high resolution image

The density of blinking dots is thus important and controlled by intensity of red laser and UV light [5].

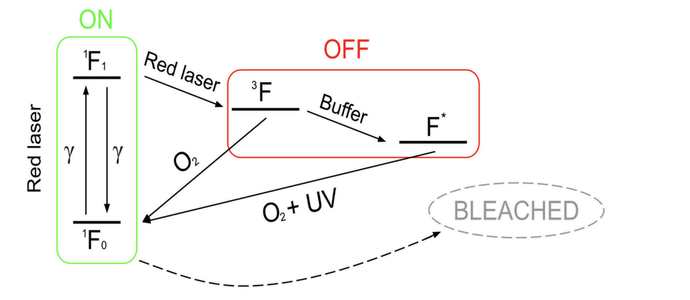

Fig. 4: Energy level diagramm of fluorophores with identification of ON, OFF and BLEACHED states

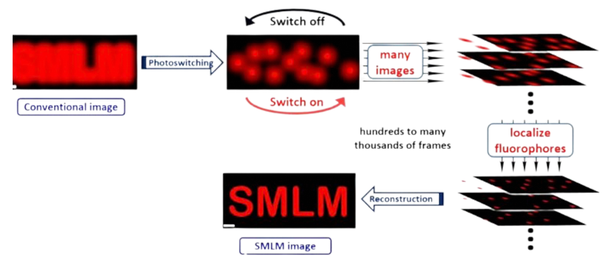

Fig. 5: Schematic presentation of the STORM imaging process [4]

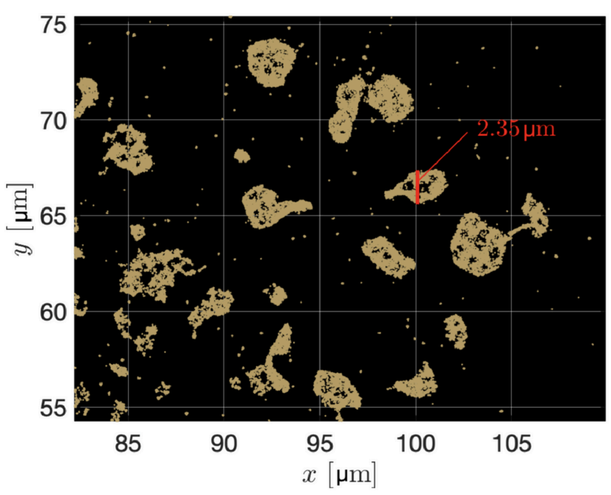

Fig. 6: Scheme [6] and STORM image of mitochondria with indication of the diameter of one of them

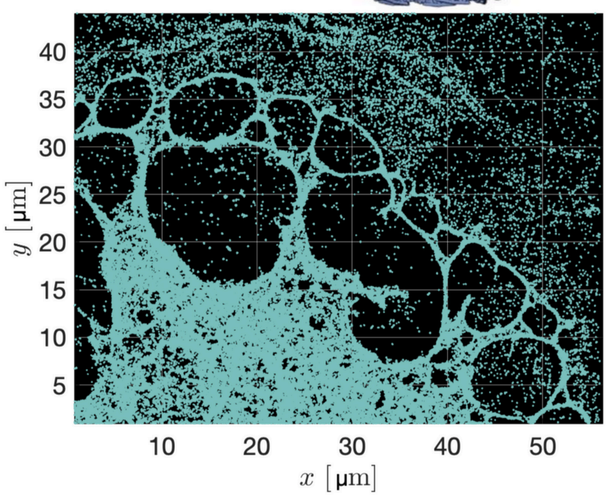

Fig. 7: Scheme [7] and STORM image of clathrin forming triskelions structures

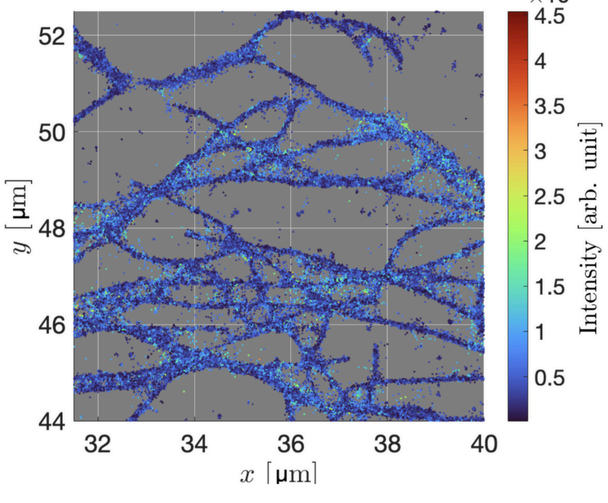

Fig. 8: Scheme [8] and STORM image of microtubules with intensity

- **Mitochondria:** Fig. 6 shows many mithochondria of the expected order of nanometers
- **Clathrin:** in Fig. 7, one can identify many triskelions which are highlighted in light blue on the scheme above
- **Microtubules:** in Fig. 8, theses hollow cylindrical polymers of tubulin proteins assemble together, leading to a complex net-work
- **Parameters :** number of frames, exposure time, lasers' intensity, ...

Conclusion

STORM imaging combined with PSF and SMLM provide a fluorescent microscopic system of high resolution, as it allows accurate study of mitochondria, clathrin and microtubules. The way SMLM and PSF techniques localise a particle through airy disk intensity profile has been found to be a reliable method, down to the nanometer. This is why each of the three microscopic biological structures can be identified, when looking at the result of the process. Thus, probabilities and superposition of images overcome the laws of diffraction !

References

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