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# Massive Parallelization of STED microscopy with optical lattices

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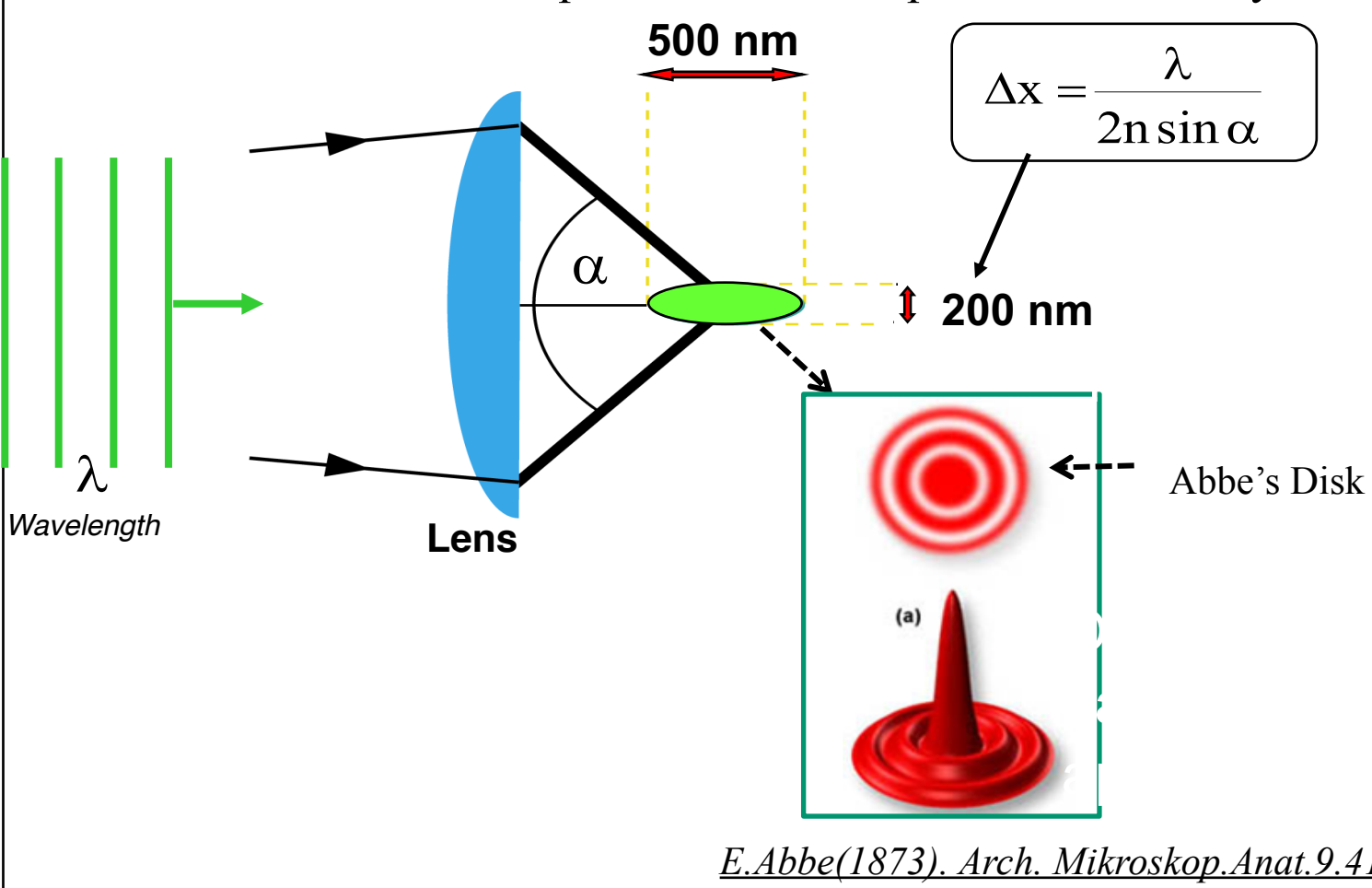
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## Introduction

### Diffraction limited optical resolution :

Due to its wave property, when light passes through an objective, it does not focalize into a point but forms a pattern called Airy disk:



Optical resolution is an important property of a microscope, often crucial for studying activities on a microscopic scale, as in biological systems and nanophysics. And it is thought to be limited by the diffraction to hundreds of nanometres.

To overcome the diffraction limit, different super-resolution methods have been proposed and realized, such as PALM, STORM, UPAIN, SIM, RESOLFT, STED<sup>(1)</sup>, .... By switching on and off the molecules consecutively, these methods push the resolution to tens of nanometres, which opens up the study of the nature in the nanometer scale.

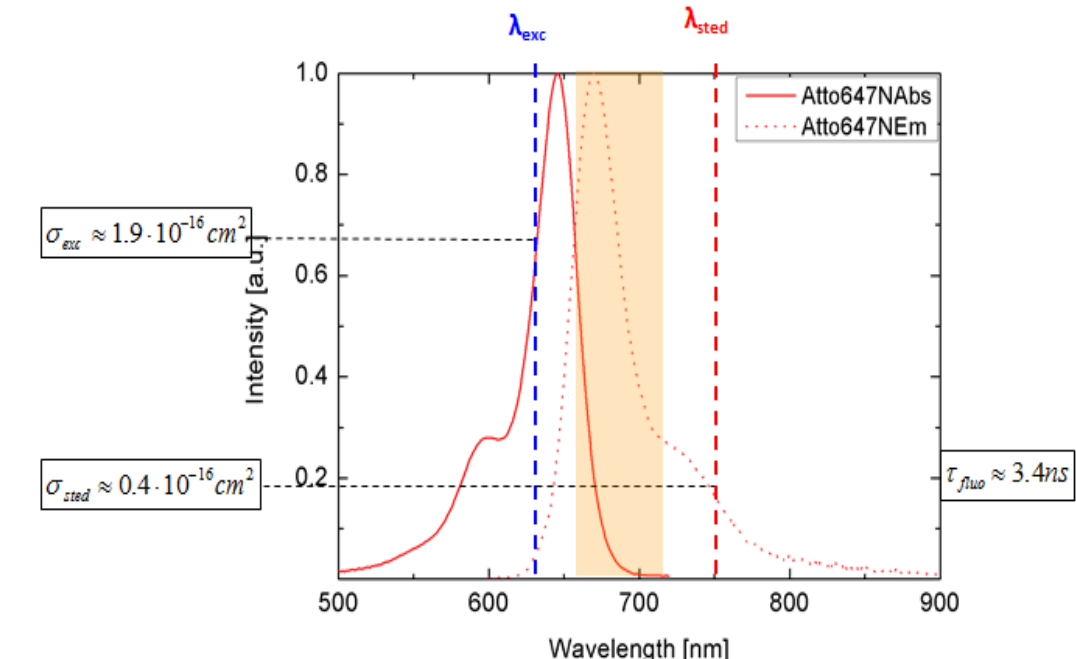
### Comparison between the super-resolution methods :

Detection methods	Parallelized detection	Single point detection
Excitation	stochastic methods and non-linear SIM	STED
Detector	Epifluorescence	Focalized spot
Field of View	Camera	APD
Speed	Large (100 μm)	Small (10 μm)
	Slow	Fast

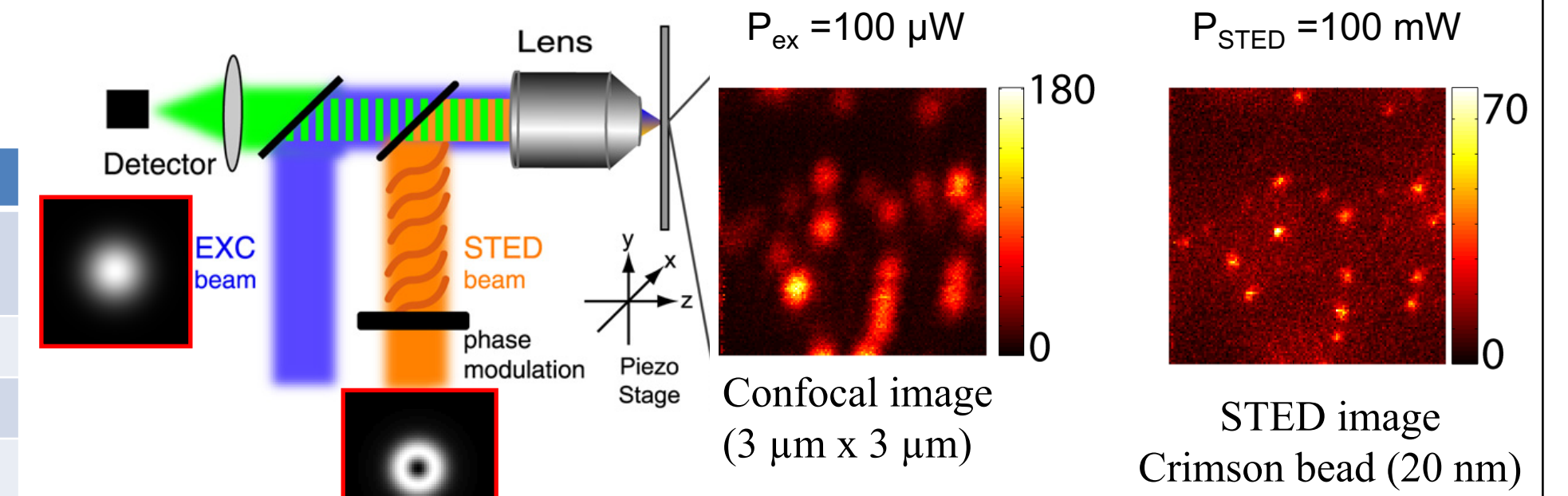
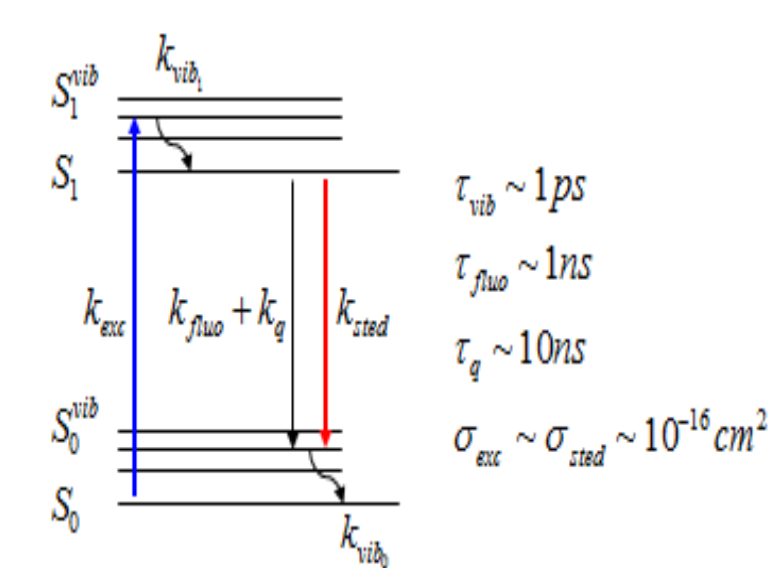
### STED Principle :

Two beams are used: one with a **Gaussian form** to switch on (excite) the molecule, the other one with a **doughnut form** to switch off (deplete) the molecule.

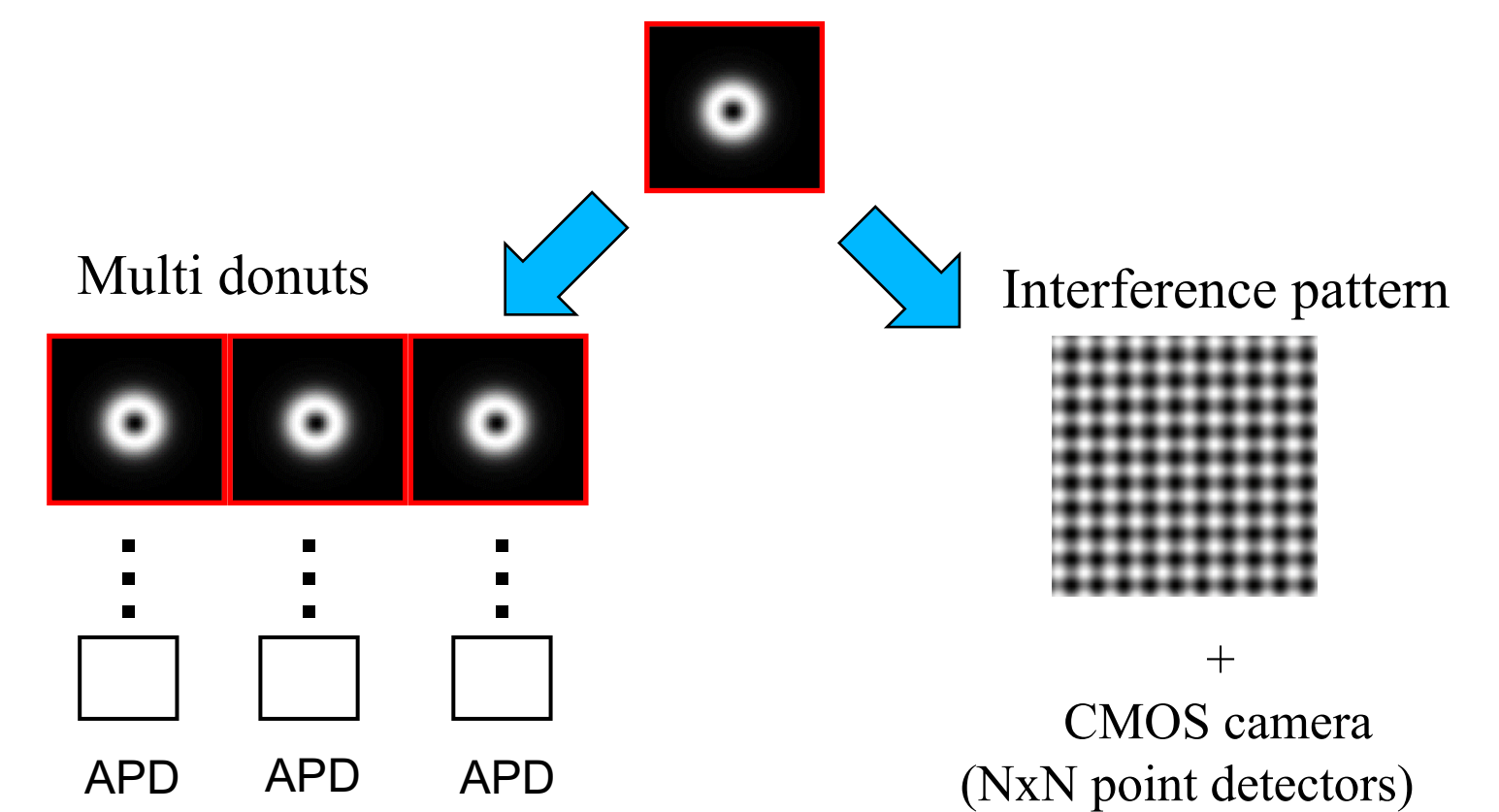
### Excitation and emission spectra of Atto647N



### Energy level diagram



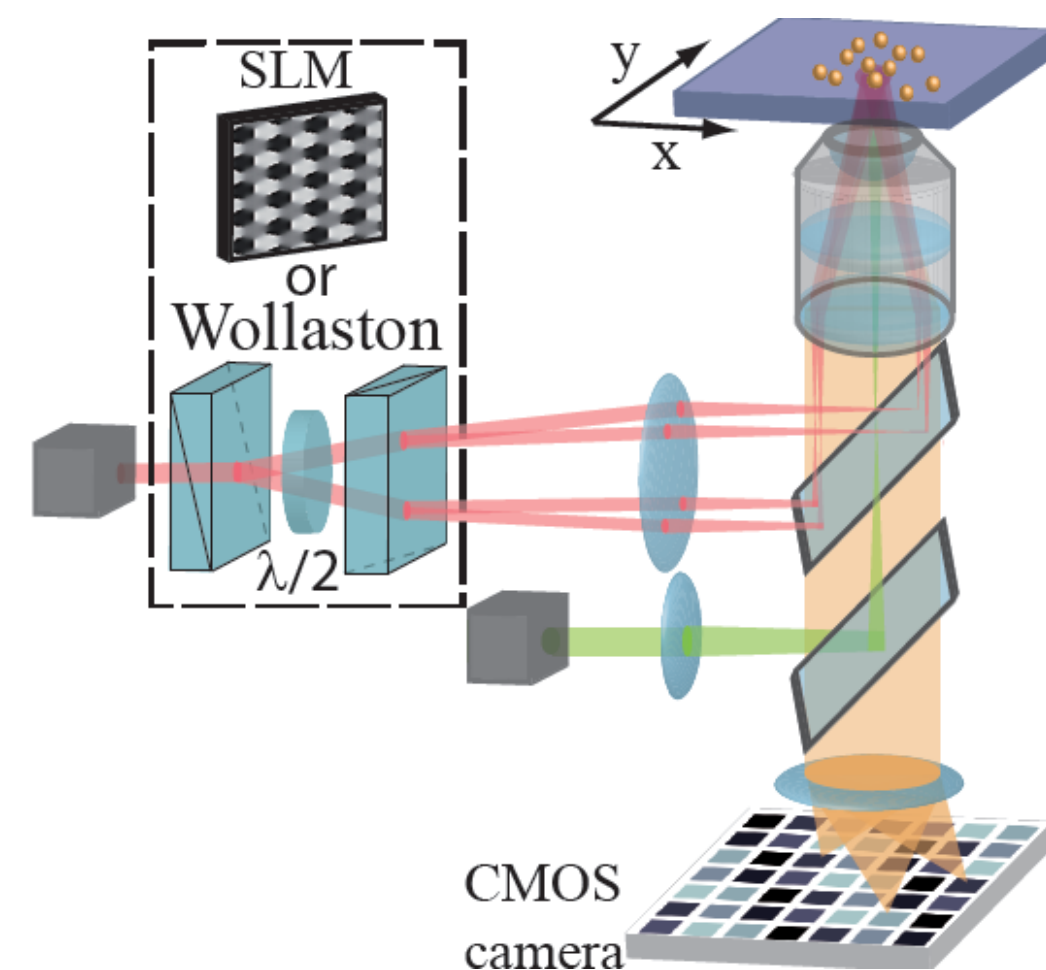
### A way for parallelization :



Multi-donuts	Interference pattern
The zeros are isolated	Intensity shared by different zeros
Few μm x few μm	a unit cell scan (300x300nm <sup>2</sup> )
	Larger intensity gradient close to min.
Hard to parallelize	Large array of zeros and detectors

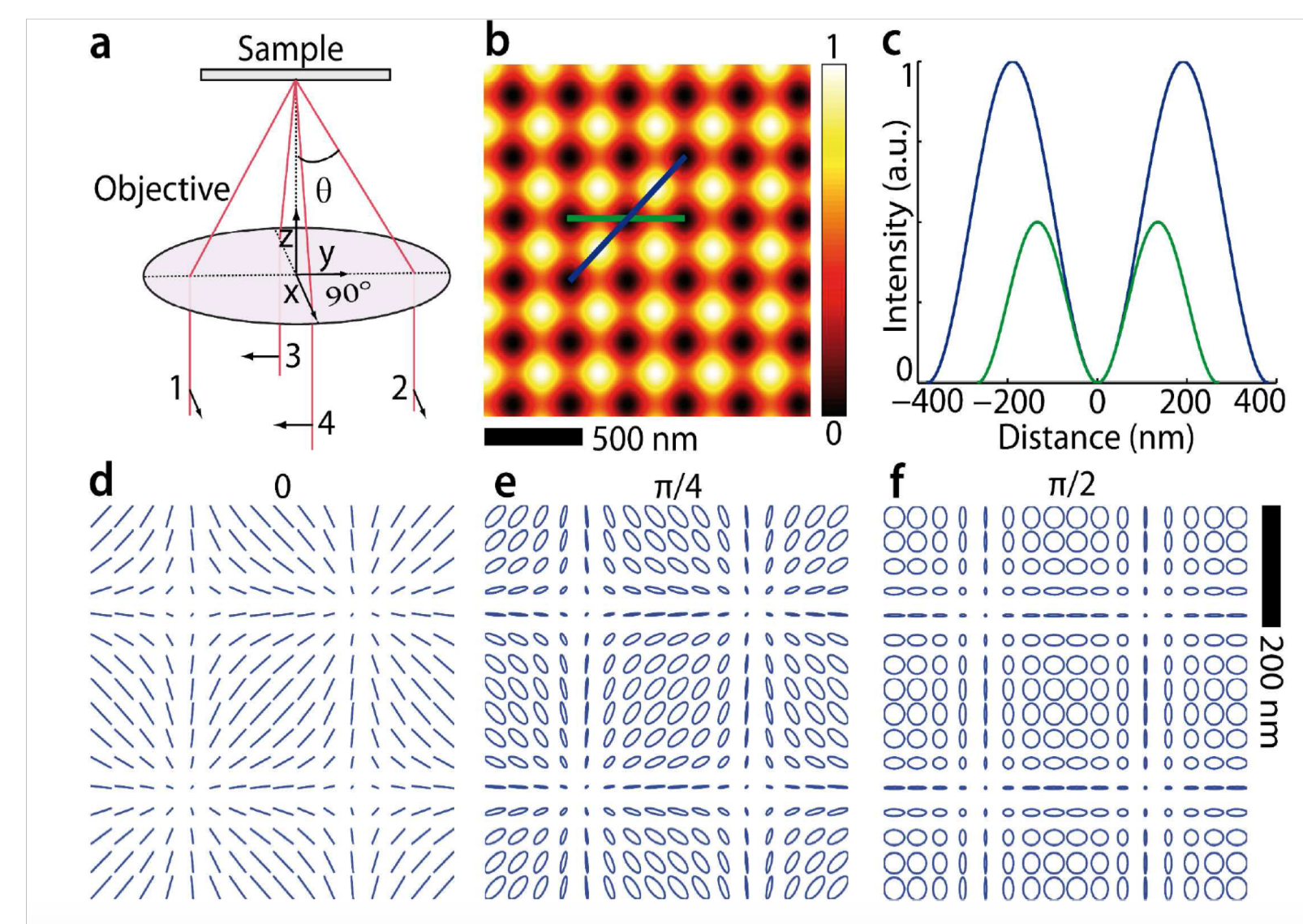
## Experimental Set-up

### Light Paths

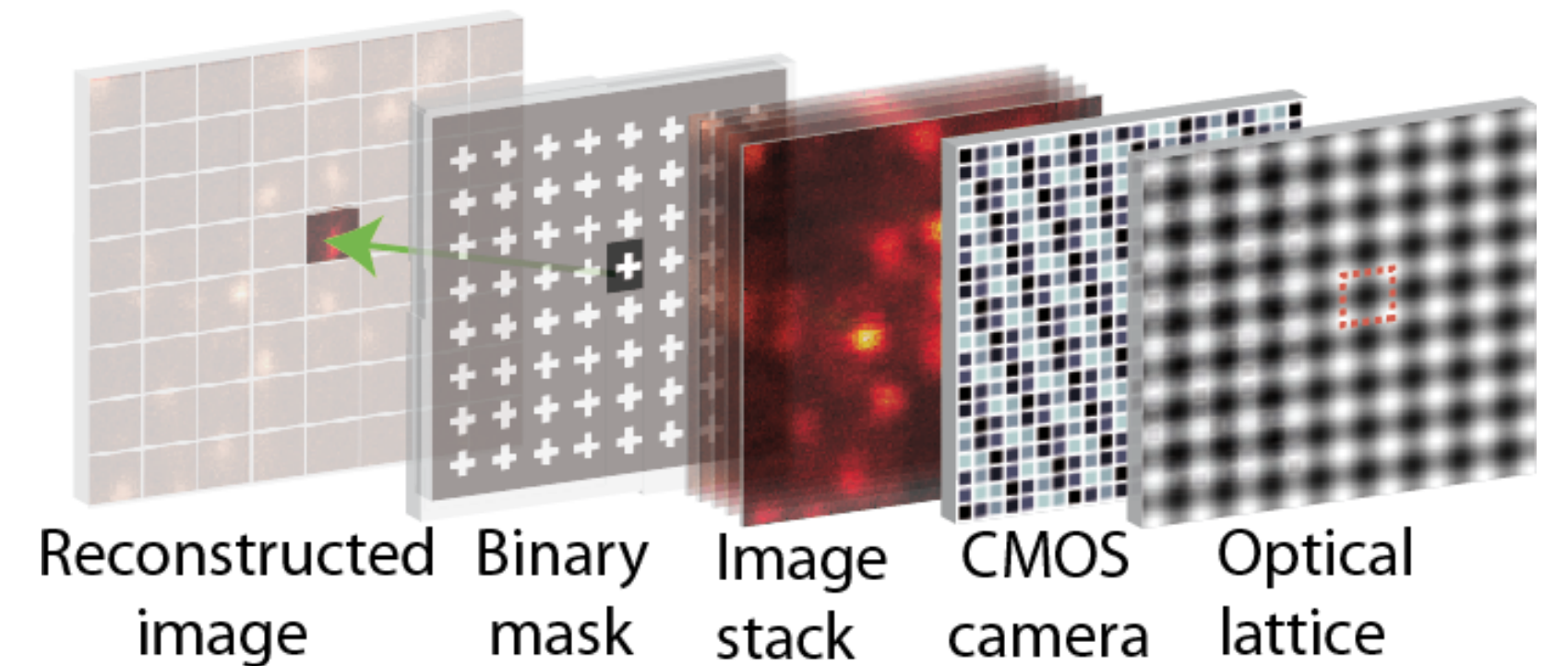


- A Spatial Light Modulator (SLM) or two Wollaston prisms generate three or four depletion beams
- An optical lattice is produced at the focal plane of an objective (NA = 1.49) and overlaid with a uniform excitation beam : Wild field illumination : 9 μm x 9 μm.
- The sample is mounted on a piezo-scanner stage to record image;
- The fluorescence is filtered from the excitation and the depletion beams, and recorded using a fast CMOS camera.

▪ A key point for faster image acquisition and better resolution: generate lattices with the smallest possible unit cell.



### Imaging processing :

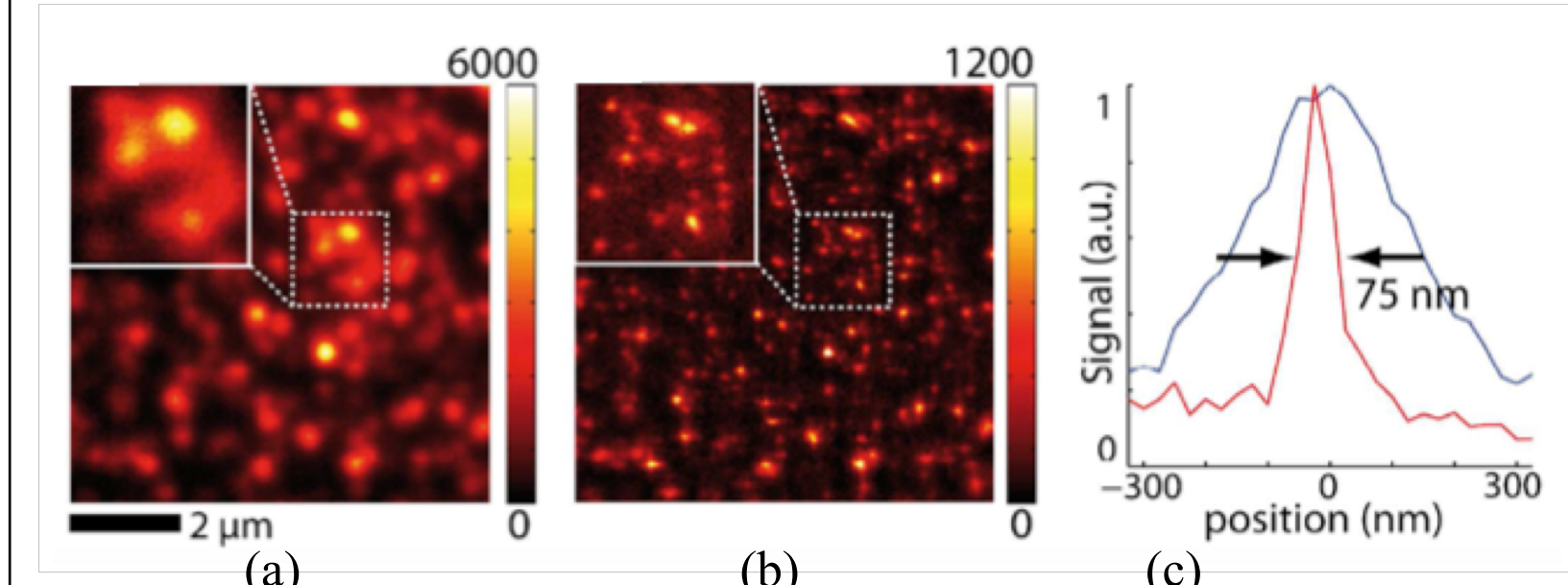


- Scan the sample over a unit cell with excitation and depletion beams,
- Acquire a fluorescence image (128\*128 pixels, 25 nm) for each scanning step.
- Apply a binary mask on the image :
  - CMOS camera + digital mask : an array of parallelized point detectors.
  - 100 parallelized detectors (13 camera pixels each)
- Each point detector records an image of one lattice unit cell (300 nm x 300 nm)
- The complete In-STED image is then obtained by juxtaposing all the unit cell images together.

## Results and perspectives

### OL-STED on fluorescent beads and cells :

- Crimson beads (diameter : 20 nm) to calibrate the Lattice-STED resolution.
- Excitation power : P = 16 mW
- Depletion power : P = 850 mW

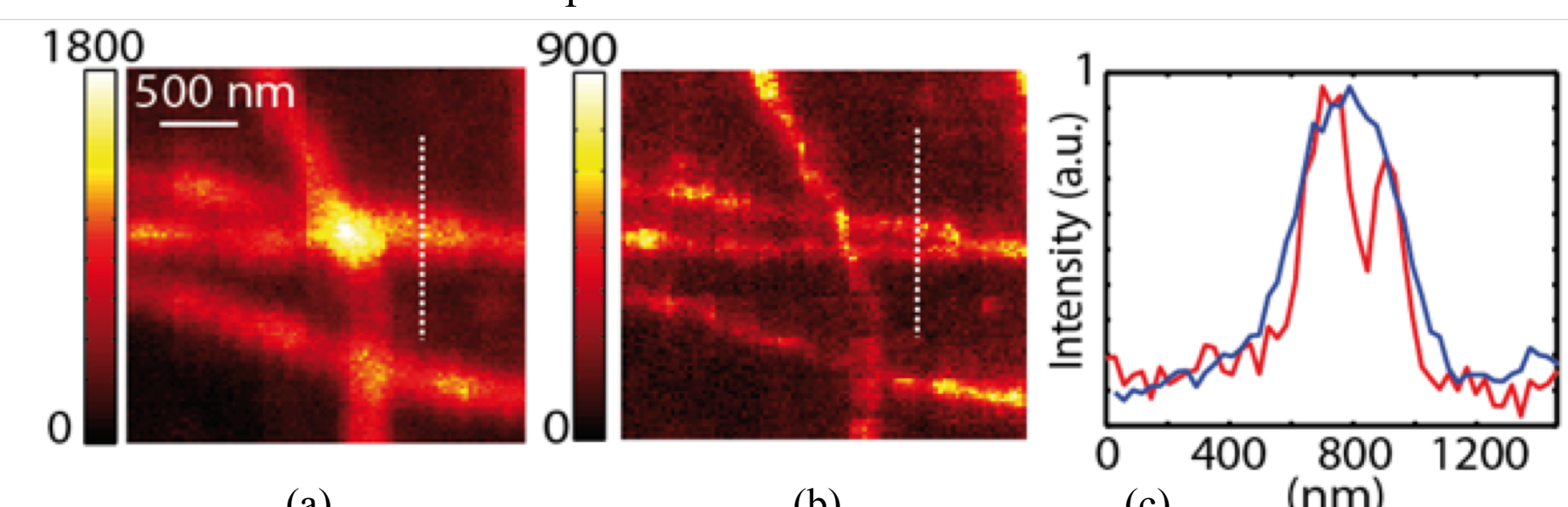


Fluorescent Crimson beads. (a) confocal image; (b) OL-STED image; (c) profiles along the dashed lines; pixel dwell time: 20 ms.

### Results:

- Achievable resolution down to 75 nm.
- Comparable with commercial STED microscope
- Field of view 7.5 μm x 7.5 μm.

- We tested the OL-STED with stained microtubules in COS cells (ATTO647N).
- Special conditions and requirements:
  - The depletion beam excites the sample, need to increase the excitation intensity : severe photobleaching. Short integration time 800 μs/point
  - Use of structured excitation pattern.

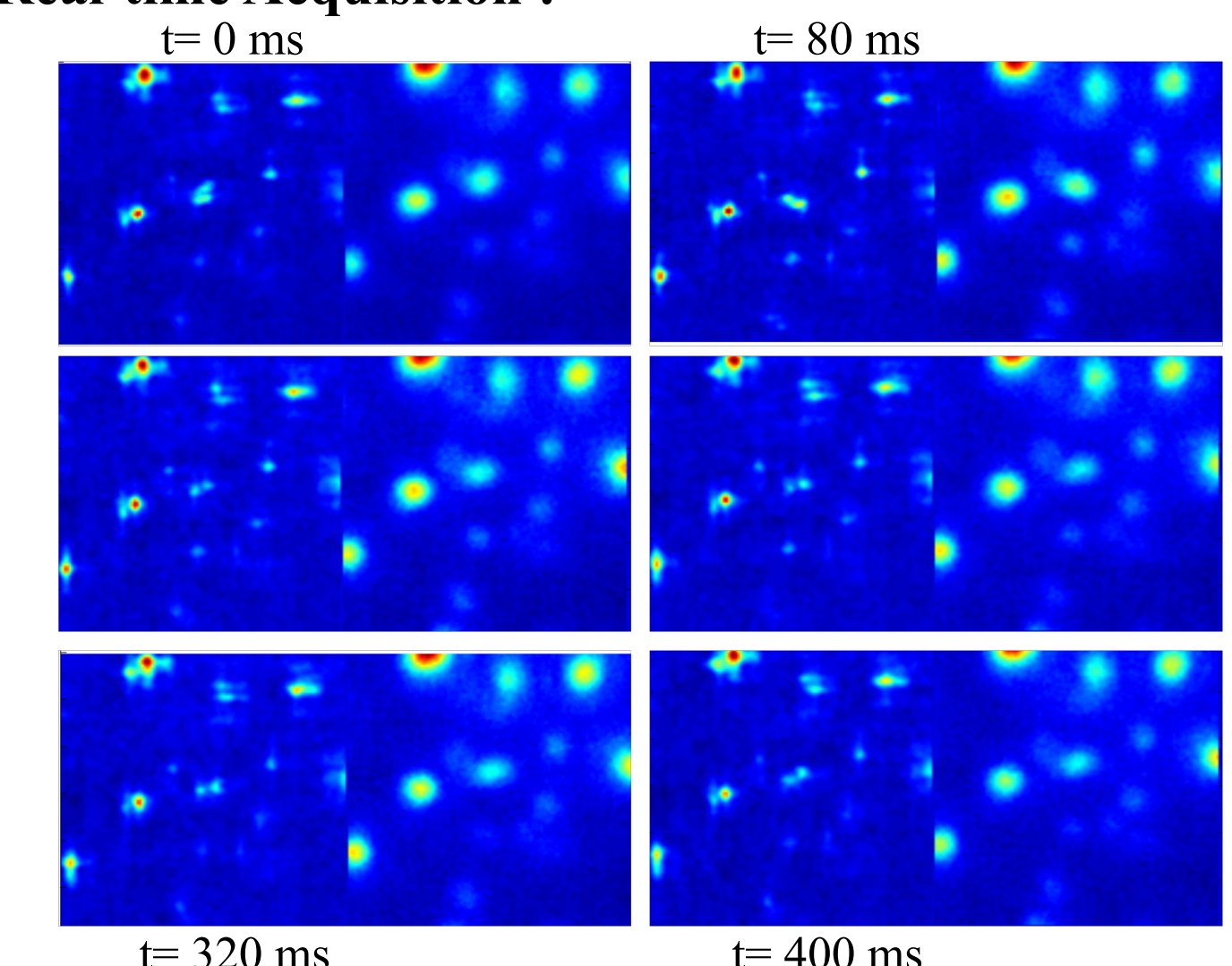


Microtubules in COS cells : (a) confocal image, (b) In-STED image; (c) profiles along the dashed lines. Acquisition time : 80 ms.

### Results:

- The OL-STED resolves structures unobservable in structures in confocal configuration.
- The microtubule super-resolved image is limited by the intrinsic microtubule's size.

### Real-time Acquisition :



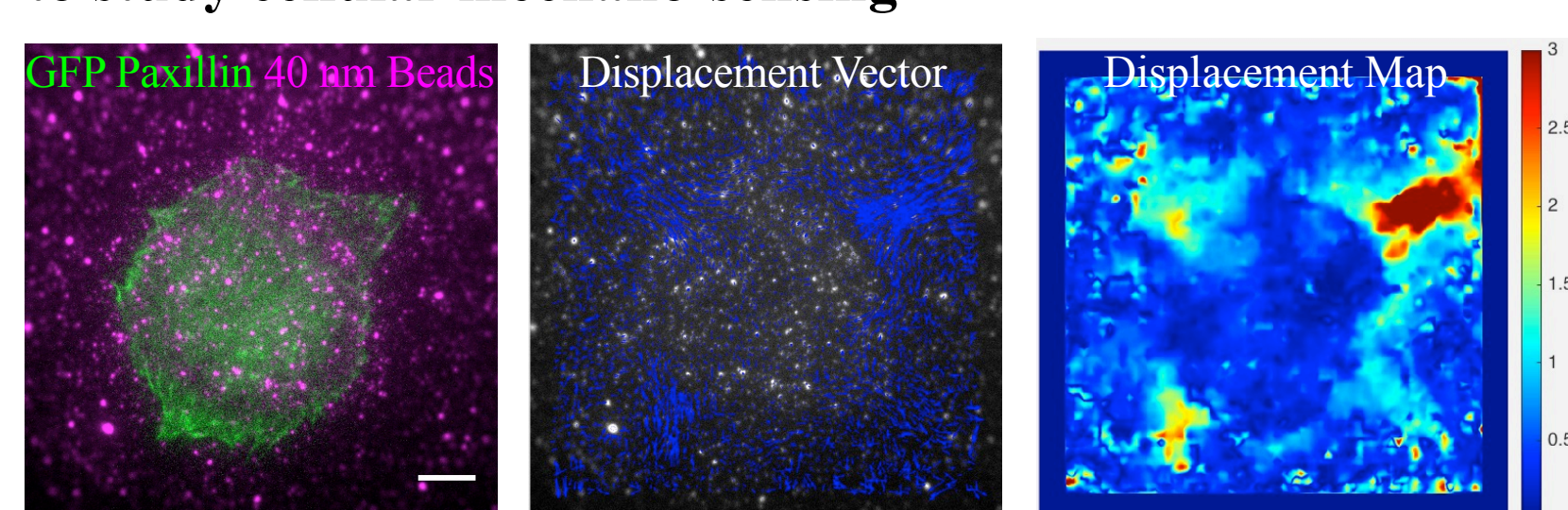
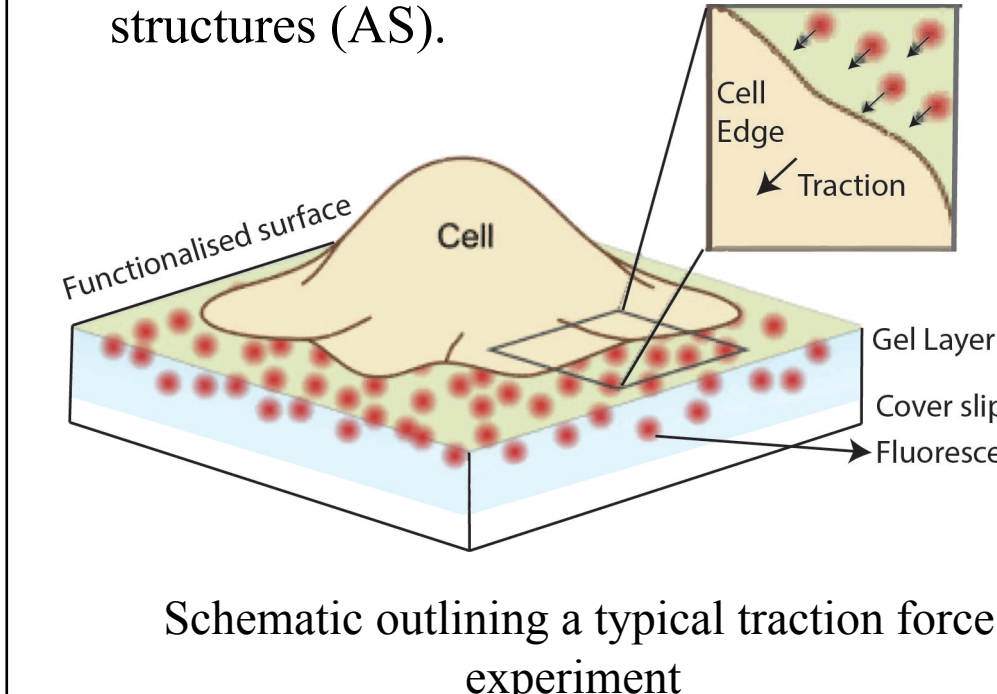
Comparison between OL-STED (left) and confocal images (right). Crimson beads in Carbol gel (2%).

### Results:

- Acquisition speed: up to 12.5 frames/second (limited by the camera readout time). Recording time : 8 s.
- Parallelized single fluorescent Crimson beads tracking.

### Perspective : using parallelized STED to study cellular mechano-sensing

➢ Conventional Traction Force Microscope (TFM) to map at the sub-cellular level forces generated by integrin-dependent adhesive structures (AS).



(left) Mouse embryonic fibroblast (MEF) expressing paxillin-GFP as an AS reporter spread on an elastic polydimethylsiloxane (PDMS) substrate (36 μm thickness, 0.7 kPa stiffness) functionalized with fibronectin, and doped with fluorescent beads (40 nm). (middle) Displacement vector (right) corresponding displacement map between 1st and 100th frame (2 frames/sec). Scalebar 10 μm.

Super resolved Traction force microscope to map at the nanoscale forces within an integrin-dependent adhesive structures (AS).

- Cells will be spread over a gel surface embedded with a high-density layer of Nano-diamonds. The density of Nano-diamonds will be high enough to sample the forces inside AS at the nanoscale level.
- Displacements of the emitting Nano-diamonds will be measured using OL-STED and 2D in-plane traction forces will be mapped.
- This strategy will allow us to record the traction forces cartography in an AS at nanoscale levels and fast rates.

### Acknowledgement :

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### References

1. B. Yang, *et al.*, "Large parallelization of STED nanoscopy using optical lattices", *Opt. Express* 22, 5581-5589 (2014).
2. B. Yang, *et al.*, "Polarization effects in lattice-STED microscopy", *Faraday Discuss.*, 2015,184, 37-49.