STED microscopy
Super resolution fluorescence microscopy

Daniel Rönnlund
danron@kth.se, 08-55378789
Biomolecular Physics, KTH-Stockholm
Course in Quantitative Microscopy 2012-11-06
Super resolution fluorescence microscopy

Part 1: Method
- Labeling / fluorophores
- Confocal and STED (stimulated emission depletion) microscopy
- Other super resolution techniques

Part 2: Image processing and analysis (for high resolution images)
- Deconvolution
- Colocalization / Nearest neighbor analysis
- Spots: size, density & location
- Structural analysis

..Please interrupt if you have questions!
Specific targets (antigens) are either labeled with fluorophores, usually done by antibody staining, or fluorescence protein (e.g. GFP) transfection.

Each fluorophore has a spectra with a separate excitation and an emission part, where the emission is shifted towards higher wavelengths.

Antibody labeling

GFP structure

Excitation

GFP spectra

Detection
Method – Confocal microscopy

There are several commercially available Confocal systems (Zeiss, Leica, Olympus …)

By adding a pinhole out of focus light is removed from the detection

Benefits of fluorescence
- High specificity (labeling) and sensitivity (detection)
- ”Easy” to prepare and label samples
- Possibility to look at live cells and into tissue
Method – Confocal microscopy

**Ernst Abbe** “objects that are too close will not be resolved” - 1873

**Lord Rayleigh** “if you want to resolve two stars they cannot be too close to each other” - 1879

Resolution $\sim \frac{\lambda}{2n \sin \theta}$

Example: For $\lambda = 600$ nm and N.A. = 1.4 $\Rightarrow$ Resolution = 214 nm
Method – Confocal/STED

Spontaneous emission

Diffraction limited focus

Excitation focus

Intensity (norm.)

250 nm

500 nm
Method – Confocal/STED

Vortex Phase Plate

d = \frac{\lambda}{2n \sin \theta \left(1 + \frac{I_{\text{max}}}{I_{\text{sat}}} \right)}
Method – Resolution and power

- 250 nm
- 150 nm
- 90 nm
- 50 nm

1 MHz
100 ps pulses

Scale bar 1 μm

More information
Method – STED at KTH

Scale bar 100 nm
Method - Alignment

**PSF's**

- **STED1**
- **STED2**
- **Exc1**
- **Exc2**

**Overlap**

**XY**

- 500 nm

**XZ**

- 500 nm

**APD's**

- X-scan
- Y-scan
- Z-scan

Normalized intensity vs. distance (nm)
Stronger demand on fluorophores.
- Stability (bleaching)
- Spectra
Method – STED example image

Fibroblast, Actin – Red, Phosphotyrosine - Green

Scale bars 2 µm
Method – Different STED techniques

Video-rate STED

Method – Different STED techniques

3D - STED

Method – Different STED techniques

STED in tissue samples and living specimen

S. Berning et al. Science (2012)

Method – Leica STED

Pulsed and CW versions available.
Resolution <60 & <80 nm respectively
Cost ~1 000 000 euro

User friendly system
Self-aligning
Multicolor-confocal / ”dual-color STED”
Method - STED

• Greatly improves resolution compared to conventional confocal / widefield microscopy. Usually 25-50 nm is achieved in the axial-plane for fluorophores and fluorescent proteins but < 5 nm has been achieved on nanodiamonds*.
• Possible to get high resolution in lateral plane (<100 nm) but puts more demands on the set-up.
• No computations needed ("Just Physics!")
• Live cell imaging possible and even live tissue imaging. Fast scanning allowing for video-rate image acquisition.

• More complex set-up required. More alignment.
• Higher demands on dye photostability and spectrum.
• High laser power demanded for best resolutions.

Method – Other super resolution techniques

RESOLFT
Reversible Saturable (Switchable) Linear Fluorescence Transitions
Same principle as STED but instead of using stimulated emission the dyes are switched off by other mechanism such as photoswitchable dyes or pushing the dyes to a dark triplet-state.

Benefits/Drawbacks
• Much lower powers needed to get high resolution
• Other dyes can be used. Many fluorescent proteins are photoswitchable.
• Resolution dependent on how many on-off switching cycles the dyes can go through. Usually slightly lower resolution compared to STED.
Structured illumination microscopy (SIM)
-use excitation grating to create moiré patterns which can show confocally unresolved details in the image

- Improves both lateral and axial resolution ~twice compared to the diffraction limit. (100 nm axial 250 nm lateral achieved)
- Easy to image multiple dyes
- Fast acquisition speeds
- Many commercially available systems (Zeiss, Leica, Nikon)

a: Sample Image  
b: Illumination Pattern 
c: Moiré pattern

Leica SIM image
Method – Other super resolution techniques

Localization microscopy
- Identify the targets one at a time and calculate their position

STORM
Stochastic Optical Reconstruction Microscopy

PALM
Photoactivated Localization Microscopy
Method – Localization microscopy

\[ \sigma \approx \frac{\sigma_{PSF}}{N^{1/2}} \]

Method – Localization microscopy

online.physics.uiuc.edu
Method – Localization microscopy
B-SC-1 cell,
Microtubules stained with anti-β tubulin
Cy3 / Alexa 647 secondary antibody
Method – Localization microscopy

• Possible to get very high spatial resolution in axial plane (10-20 nm)
• Also possible to get high resolution in lateral plane (~60 nm)
• Easier set-up compared to STED. Widefield or TIRF microscopy instead of pixel-scanning. (Cheaper)

• Long exposure time to get images (usually 10-15 minutes)
• Computations required to reconstruct a high resolution image from large image stacks, usually >10 000 images needed. Sensitive to drift during capture.
• Difficult to get multicolor images due to specific demands on fluorophores.

Commercially available systems: Nikon & Zeiss
(Zeiss ~1 000 000 euro, <50nm resolution STORM ~100nm SIM)
Summary Super Resolution Techniques

• Many techniques available to surpass Abbes diffraction limit, each with their own benefits and drawbacks.
• STED/RESOLFT provides the high resolution images directly without any need for computations, fast-image acquisition and 3D imaging possible as well as to look into live tissue. Drawbacks are more complex microscope design and higher demands on photostability for dyes.
• SIM is well established and cheaper, it puts less demands on dyes (although photostability is still important) and multiple colors are easier to get. Drawbacks are the relative low resolution improvement.
• PALM/STORM provides very high resolution and does not demand a complex microscope design (although stability is very important). The major drawback is the need to take large image stacks to get the highest resolution which is usually a slow process.

(There are many other techniques available which I have not talked about, some examples are SOFI, SHRImP and FIONA)
Part 2: Image processing and analysis

“"A picture is worth more than a thousand words”"

What do the images tell us?
Higher resolution $\rightarrow$ more information
How do we extract the data?
Image processing - Deconvolution

A microscope image is a ”blurred” version of the true sample

By prior knowledge about how the PSF looks like we can enhance or ”deblur” the image

• Greatly improves contrast and reduce noise in the image
• Slightly increases resolution
→ Makes image analysis easier!

AutoQuant X: Deconvolution & 3D Visualization Imaging Software
Image processing - Deconvolution

Confocal

STED

Deconvoluted STED

Platelet, VEGF (Green), Actin (Red)

Scale bar 1 µm
Image analysis – Colocalization

Colocalization is the intensity overlap of different labeled targets in an image. If green and red intensity colors are used then yellow symbolize colocalization.

Common colocalization coefficients to use are Manders and Pearson:

\[ M = \frac{\sum G \times R}{\sum G \times \sum R} \]

\[ P = \frac{\sum (G - \bar{G}) \times (R - \bar{R})}{\sum(|G - \bar{G}|) \times \sum(|R - \bar{R}|)} \]

Neuron cell labeled for the sodium-potassium pump (NKA) shown in green and dopamin receptor (D1R) shown in red.

Blom et al, BMC Neuroscience, 2011
Blom et al, Journal of Microscopy and Technique, 2011
Nearest Neighbor analysis a good alternative at higher resolutions (when you start to see the individual proteins / protein clusters) to measure the distances between the labeled targets.
Image analysis – Nearest Neighbor
Image analysis – Nearest Neighbor

Fibroblast labeled for actin (red) and phosphotyrosine (green).

Green = all spots which have at least 4 neighboring spots within 300 nm
Antibody size may affect colocalization coefficients especially at high resolutions.

Nearest Neighbor analysis will give same average but broader distribution.
Image analysis – Spot Size & Density

Platelet, VEGF (Green), Actin (Red)
Size distribution, average size: 69 ± 13 nm
Amount of Spots: 107 (9.8 / µm²)
Spots per zone:
17.76 % | 28.97 % | 25.23 % | 18.69 % | 9.35 %

Gain information regarding how proteins are stored, where they are stored and how many there are.
Image analysis – Spot Size & Density

**VEGF**

- Control
- Thrombin
- ADP

**PF4**

- Control
- Thrombin
- ADP

Image analysis – Structure features

1. Entanglement
2. Squiggles
3. Direction
4. Curvature

Fibroblast cell, Vimentin (Green), Actin (Red)
Image analysis – Entanglement

Scale bars 1 µm
Image analysis – Direction

- Peak angle ± 10°
  - a3: 0.99
  - b3: 0.27
  - 0°: 0.96
  - 5°: 0.60
  - 10°: 0.38
  - 30°: 0.18

[Diagram showing normalized distribution and images with marked directions]
Image analysis – Structure example

BJ

BJ-metastatic

STED
Confocal

STED
Confocal

Scale bars 2 µm

Rönnlund et al, in prep
Summary Image Processing and Analysis

• High resolution images contain much more information which allows for deeper understanding of biological samples. Image processing and analysis provides a means to extract this information and give quantitative data.
• Nearest neighbor analysis is usually a better tool to detect how close two targets are as compared to colocalization calculations when the resolution is high enough to detect individual targets.
• When the resolution is high enough information regarding the size and density of targets are possible to get. Also specific data regarding their localization becomes available.
• More complex structures such as cytoskeletal networks can also be studied and parameters such as entanglement or direction can be obtained at higher precision.