Morning section on single molecule localization based super-resolution microscopy (SMLM)

Prezentation: general intro to SMLM (9:00-10:00)
Lab visit on hardware, software and applications (2×45 minutes 10:30-12:00)
Principles of localisation based super-resolution microscopy

Miklós Erdélyi

University of Szeged
Layout of the introductory presentation

Diffraction limited imaging

• The point spread function
• Rayleigh resolution limit
• Image formation of a wide-field microscope

From diffraction limited imaging to localization based super-resolution microscopy

• Localisation based super-resolution microscopy
• Examples
• Issues and artefacts
Diffraction limited imaging - Point Spread Function

Object: point-like source
Imaging system: diffraction limited

Diffraction limited point spread function = Airy pattern

Cylindrical symmetry

Ring system

FWHM(\(\lambda, \text{NA}\))
Two diffraction spots can be resolved, if their separation is larger than the distance between the main peak and the first minimum.

In the limiting case the minimum between the two peaks is approximately 80% of the main peaks.
Imaging of extended 2D objects

Measured Image = distribution of dye molecules $\otimes$ PSF

Measured Image = $x \otimes$
Definition of spatial resolution

How can spatial resolution be enhanced?
• Wavelength reduction (autofluorescence, optics)
• Using high NA lenses (immersion objectives)
• Manipulation of the $k_1$ factor

$R = k_1 \cdot \frac{\lambda}{\text{NA}}$

But PSF engineering typically introduces significant loss of light!

IN THE VISIBLE REGION: $R \approx 250$-$300$ nm
Microscopy methods

- Scanning/wide field
- Transmission/reflection
- Bright field/dark field
- Optical/electron
- Fluorescence/non fluorescence
- Intensity/polarization
- Diffraction limited/super-resolution
  
  Localization microscopy: Optical, super-resolution, wide-field and fluorescence method
Super-resolution methods in microscopy

Optical Super-resolution Methods
### PSFs in different microscopic methods

<table>
<thead>
<tr>
<th></th>
<th>CLSM</th>
<th>STED</th>
<th>CW-STED</th>
<th>3D-SIM</th>
<th>PALM/STORM</th>
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<tbody>
<tr>
<td>( \lambda_{\text{em}} ) [nm]</td>
<td>460-670</td>
<td>670</td>
<td>520</td>
<td>620</td>
<td>520</td>
</tr>
<tr>
<td>( D_{xy} ) [nm]</td>
<td>180-250</td>
<td>60</td>
<td>70</td>
<td>130</td>
<td>110</td>
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<tr>
<td>( D_z ) [nm]</td>
<td>500-700</td>
<td>700</td>
<td>560</td>
<td>340</td>
<td>280</td>
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<tr>
<td>( V_{xyz} ) [(10^{-3}) (\mu\text{m}^3)]</td>
<td>10-23</td>
<td>1.3</td>
<td>1.5</td>
<td>3.0</td>
<td>1.8</td>
</tr>
</tbody>
</table>
Fluorescence microscopy

Point-like source = fluorescent dye molecule

High specificity!
Optical Fluorescence Microscopy – Multicolour –

Convallaria cross section.
3 channel autofluorescence.
Supercontinuum (“white laser”) light illumination.
[ Laser Analytics group 2010 ]

A human cancer cell dividing.
3 Fluorescent labels:
  DAPI (stains DNA) – blue;
  INCENP (a protein) – green;
  Tubulin (cell skeleton) – red.
[ F. Lamiot, 2010 ]
A single fluorescent molecule is a point-like light source. Its image is blurred. (And pixelated.) The centre of the fitted curve corresponds to the position of the molecule. (Accuracy depends on the signal to noise ratio.)
Localization procedure

Collection of image stacks → Segmentation → Fitting → Judging, selection of true localization → Storage, Registration

- Definition of spatial resolution
- Localization procedure
- Segmentation
- Storage, Registration

Visualization
**Principles of the localization method - densely labelled sample -**

Object

Aerial Image

Pixelated Image

2 µm

**FWHM**<sub>PSF</sub> = 220 nm

Pixel size = 160 nm
However, if a sparse subset of molecules can be switched on …

... 2000 simulated frames

Simulated Images
... then exact positions of many molecules can be found!
Gaussian fitting is applied because:
- It is simple and fast (coordinates can be separated)
- Only the peak position is important ($a \approx s$ => substructure cannot be resolved)
- It is very close to the exact PSF (vector diffraction at high NA)

Fig. 1. Dipole orientation averaged PSF for a 100×/1.25 water immersion objective, an Airy-distribution for the same NA, and a Gaussian with standard deviation $\sigma = 0.25\lambda/\text{NA}$. 
Localization precision (photon number)

\[ \langle (\Delta x)^2 \rangle = \frac{s^2 + a^2/12}{N} + \frac{8 \pi s^4 b^2}{a^2 N^2} \]

- **N**: photons
- **s**: PSF size
- **a**: pixel size
- **b**: background noise

Number of photons
Fluorescent background reduction: EPI versus TIRF illumination

- Excitation beam is focused into the centre of the back focal plane
- The whole volume of the sample is excited
- Sectioning is limited by DOF ($\propto \lambda/NA^2$)
- High fluorescence background
Fluorescent background reduction: EPI versus TIRF illumination

EPI

TIRF

405 nm

488 nm
Fluorescent background reduction: EPI versus TIRF illumination

- Excitation beam is focused on the edge of the back focal plane
- Very thin section of the sample is excited
- Sectioning is limited by the penetration depth of the evanescent field (<150nm)
- Reduced fluorescence background
Penetration depth in TIRF mode

Wavelength = 450 nm
Glass-water surface

Normalized intensity vs. Penetration depth [nm]

1/e threshold
Laser illumination (excitation) of the sample: EPI versus TIRF

See more during the lab visit!!
Localization microscopy: a five-step experiment

1. Find a switchable fluorophore
2. Label some living cells
3. Capture ~10,000 images
4. Localize fluorophores
5. Reconstruct Hi-res image

**PALM, STORM, dSTORM, FPALM, FIONA, GSDIM**
Photoactivated localization microscopy (PALM)
**Photoactivated localization microscopy (PALM)**

<table>
<thead>
<tr>
<th>Readout laser (488 nm)</th>
<th>Activation laser (405 nm)</th>
</tr>
</thead>
</table>

**Diagram:**
- Readout laser (488 nm) affects the image in different ways depending on the illumination volume.
- Activation laser (405 nm) also influences the image.

**Images:**
- **A:** Ar+ illumination volume
- **B:** 405 illumination volume
- **C:** Image with combined illumination
- **D:** Image with crosshair lines
- **E:** Image with crosshair lines and red crosses
- **F:** Image with crosshair lines and red crosses
1. All fluorophores are initially “switched off”
2. Activate sparsely with UV
3. Image sparse dyes until they bleach
4. Repeat steps 2 and 3

**Sparse fluorescence by photoactivation**

- Dark state
- Bright state
- Bleached state
**Definition of spatial resolution**

Photoactivatable fluorescent proteins

**Advantages:**
- High labelling specificity
- Life-cell imaging

**Limitations:**
- Low quantum efficiency
- Complicated labelling procedure

The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, William E. Moerner and Stefan W. Hell "for the development of super-resolved fluorescence microscopy".

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### List of PAFPs

<table>
<thead>
<tr>
<th>PAFP</th>
<th>Absorbance$_1$ (nm)</th>
<th>Emission$_1$ (nm)</th>
<th>Absorbance$_2$ (nm)</th>
<th>Emission$_2$ (nm)</th>
<th>Photoconversion wavelength</th>
<th>Reversibility</th>
<th>Brightness$_1^*$</th>
<th>Brightness$_2^*$</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Kaede (protein)</td>
<td>508</td>
<td>518</td>
<td>572</td>
<td>580</td>
<td>ultraviolet</td>
<td>none</td>
<td>2.64X</td>
<td>0.60X</td>
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<tr>
<td>Eos (protein)</td>
<td>506</td>
<td>516</td>
<td>571</td>
<td>581</td>
<td>ultraviolet</td>
<td>none</td>
<td>1.30X</td>
<td>0.70X</td>
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<td>488</td>
<td>515</td>
<td>551</td>
<td>580</td>
<td>ultraviolet</td>
<td>none</td>
<td>0.66X</td>
<td>0.49X</td>
<td>[6]</td>
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<td>516</td>
<td>390</td>
<td>?</td>
<td>490 nm</td>
<td>reversible, 390 nm</td>
<td>?</td>
<td>?</td>
<td>idem</td>
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<tr>
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<td>440</td>
<td>?</td>
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<td>reversible, 440 nm</td>
<td>?</td>
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<td>idem</td>
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<td>468</td>
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<td>511</td>
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<td>600</td>
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<td>0.13X</td>
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</table>

*Brightness values are relative to EGFP.*
Direct stochastic optical reconstruction microscopy (dSTORM)
1. All fluorophores are initially “switched on”
2. Triplet quenching
3. Slow reactivation
4. ... Sparse “on” population

**Sparse fluorescence by quenching**

**Initial State**
- All “ON”
- Triplet State (rare decay)
- Chemically quenched state

**Equilibrium State**
- Sparse “ON”
- “OFF”
- Fast
- Slow

1-5 kW/cm²
## STORM dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>Excitation Maximum (nm)</th>
<th>Emission Maximum (nm)</th>
<th>Extinction (M⁻¹ cm⁻¹)</th>
<th>Quantum Yield</th>
<th>Detected Photons Per Cycle</th>
<th>Equilibrium Duty Cycle</th>
<th>Survival Fraction (400 s)</th>
<th># Switching Cycles (Mean)</th>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>MEA</td>
<td>βME</td>
<td>MEA</td>
<td>βME</td>
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<tr>
<td><strong>Blue-absorbing</strong></td>
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<td></td>
<td></td>
<td></td>
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<td>Atto 488</td>
<td>501</td>
<td>523</td>
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<td>0.0022</td>
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<td>Alexa 488</td>
<td>495</td>
<td>519</td>
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<td>516</td>
<td>538</td>
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<td>506</td>
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<td><strong>Yellow-absorbing</strong></td>
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<td>Cy3B</td>
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<td>578</td>
<td>603</td>
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<td>0.69</td>
<td>2,826,1,686</td>
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<td>TAMRA</td>
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<td>575</td>
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<td>Cy3</td>
<td>550</td>
<td>570</td>
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<td>0.0003</td>
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<td><strong>Red-absorbing</strong></td>
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<td>Alexa 647</td>
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<td>665</td>
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<td>1,656,987</td>
<td>0.0019</td>
<td>0.0024</td>
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</table>
Clathrin mediated endocytosis (EGF labelled by Alexa 647)
Aβ_{42} fibrils in vitro (labelled by Alexa 647)
**Aβ₄₂ fibrils in cell (labelled by Alexa 647)**

In Situ Measurements of the Formation and Morphology of Intracellular β-Amyloid Fibrils by Super-Resolution Fluorescence Imaging

Gabriele S. Kaminski Schierle,¹ Sébastien van de Linde,¹ Miklós Erdélyi,¹,¹⁺ Elin K. Esbjörner,⁶ Teresa Klein,⁶ Eric Rees,¹ Carlos W. Bertoncini,¹⁺ Christopher M. Dobson,⁹ Markus Sauer,⁺⁺ and Clemens F. Kaminski⁺⁺,⁺¹
Microtubules in cells
Issues - labelling -
**Issues - labelling -**

*Short linkers and small dye molecules are required!*
**Issues - labelling density**

- **Low labelling density**
  - True localization
  - False localization

- **High labelling density**

**Definition of spatial resolution**
Issues - mechanical drift -

260nm correction

Sample: fluorescent beads
Sampling frequency: 1 frame /s
Duration: 20min
Autofocus system using a separated illumination port (Nikon PFS)


**LF405/488/561/635-4X-A-000**

- In Nikon this is a separated channel from the excitation path (cannot be directly implemented into IX71 frame)
- Requires a dichroic mirror that reflect at the applied wavelength (around 870nm)

Under ideal circumstances, the focusing precision of the PFS is usually less than one third of the objective focal depth. (http://www.microscopyu.com/articles/livecellimaging/perfectfocus.html)

See more during the lab visit!!
Definition of spatial resolution

Issues - chromatic optical offset -

(CCD pixel)
Super-resolution microscopy roadmap

Contents

Optical nanoscopy: the road ahead

Super resolution microscopy by stochastic switching of single fluorescent molecules

Structured illumination microscopy

The prospects of adaptive optics for super-resolution microscopy

Correlative super-resolution optical and electron microscopy roadmap

Microscopy reference samples produces by DNA nanotechnology

Super-resolution microscopy to dissect plasma membrane organization

Photoswitchable fluorescent proteins for nanoscopy

Drop down the light: RESOLFT microscopy in living cells and tissues

Super-resolution microscopy in neurosciences: zoom in on synapses

Nanobodies for high-resolution imaging

Super-resolution imaging of early signaling events at the leukocyte cell-surface

In vivo super-resolution microscopy

Advance image deconvolution in STED microscopy

The limiting factor: fluorescent probes for optical super-resolution microscopy

References
• Geometrical parameters

<table>
<thead>
<tr>
<th>Aligned and averaged H-zone components</th>
<th>Aligned and averaged I-band components</th>
</tr>
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<tbody>
<tr>
<td>Fhox</td>
<td>Myosin S2</td>
</tr>
<tr>
<td>SAR3</td>
<td>Protein (P3)</td>
</tr>
<tr>
<td>Tropomodulin</td>
<td>Ketin (Ig10)</td>
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<td>DAAM (R1)</td>
<td>Filamin C-termin</td>
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<td>SL5700 (R1)</td>
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<td>Actin (C4)</td>
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<td>TropomysinH</td>
<td>Projectin (Ig20)</td>
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<td>Zomin</td>
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</table>

- Number of target molecules

![Image of sarcomere structure of flight muscle (Drosophila)]

- Geometrical parameters

- Number of target molecules

![Images of NT, U2OS T, and DlvA T]
Conclusions

• The image of a point-like source (PSF) has spatial extent because of diffraction
• Spatial resolution is limited by the wavelength and NA
• Sub-diffraction imaging requires special super-resolution methods
• Super-resolution can be achieved via localization method
• Switchable fluorescent molecules are required for STORM
• Special localization algorithm is required
• Imaging parameters are critical
• Implementation of super-resolved images is critical
Definition of spatial resolution

For more details: Lab visit

optical system  sample preparation  image acquisition

HARDWARE

SOFTWARE

test sample generator  localization code  quantitative evaluation

APPLICATIONS

Thank you and see you in the lab!