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Polarization resolved super-resolution microscopy
Polar-STORM

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Outline

- Diffraction barrier
  - Super-resolution technique D-STORM
- Molecular orientational order
- Analysis of orientation of single molecules.
- Anisotropy of freely rotating single molecules
- SPIM-STORM
Diffraction barrier

- **Eye**: ~ 50-100 µm
- **1595, Zaccharias and Hans Janssen**: First microscope, 9x magnification
- **Antony Van Leeuwenhoek** (1632-1723), 200x magnification
- **Ernst Abbe** (1840-1905): The “physical” diffraction limit
- **Confocal microscopy** (1957)
- **Near-field scanning optical microscopy** (1972/1984)
- **Multiphoton microscopy** (1990)
- **4-Pi microscopy / L²M** (1991-1995)
- **Structured illumination microscopy** (2000)

**Diffraction limit:**
- ~ 250 nm lateral
- ~ 600 nm axial

Mathematical formula:

$$d \approx \frac{\lambda}{2 \ NA}$$
Breaking the diffraction limit

Fluorophore!
Super-resolution imaging approaches

Methods that use spatially patterned illumination to sharpen the point-spread function of the microscope

- Stimulated emission depletion (STED) microscopy
- Saturated structured-illumination microscopy (SSIM)

Method based on the high-precision localization of individual fluorescent molecules.

- Stochastic Optical Reconstruction microscopy (STORM)
- (Fluorescence) photoactivation localization microscopy ((F)PALM)
Localization of individual fluorescent molecules approach

The greater the number of photons -> the better the 2-D Gaussian fit

\[ d = \frac{1}{\sqrt{N}} \cdot \frac{\lambda}{2 \cdot NA} \]

Apply 2D Gaussian least squares fit to find center of centroid of point-spread-function

Yildiz et al., Science, 2003
Super-resolution imaging by localization

ST_{ochastic} \ O_{ptical} \ R_{econstruction} \ M_{icroscopy} = \text{STORM}

1. Target structure
2. Localizing activated probes
3. Storm Image

Also named as PALM (Betzig et al., Science, 2006) and FPALM (Hess et al., Biophys. J. 2006)
Example

(Henriques, 2009)
D-STORM

Actin labelled(atto655) in COS-7 cells.
Molecular orientational order
Molecular orientational order

Increasing disorder due to
- Spatial rearrangements
- Dynamics
Ensemble

Sample plane

Single molecule

Sample plane

Excitation volume

~ 300nm
Probing orientational behavior using polarized fluorescence

Free rotational motion: depolarization effect

Rotational diffusion time: \( \tau_{\text{rot}} = \frac{\eta V}{RT} \)

- Viscosity
- Molecular volume
- Temperature

\( \tau_{\text{rot}} \ll \tau_f \)
Constraint rotational motion: orientational effects

Rotational diffusion

Initial position

Final position at time $t$

Rotational constraint: $\Psi$

Dynamics:

$\tau_{rot} = \frac{\eta V}{RT}$
Probing orientational behavior using polarized fluorescence

\[ I \propto P_{\text{abs}} \cdot P_{\text{em}} \]

- \( P_{\text{abs}} \): Absorption probability of a single molecule.
- \( P_{\text{em}} \): Emission probability of a single molecule.

\[ P_{\text{abs}} \propto \left| \vec{\mu}_{\text{abs}} \cdot \vec{E}_{\text{inc}} \right|^2 \]
- \( \vec{\mu}_{\text{abs}} \): Transition dipole moment
- \( \vec{E}_{\text{inc}} \): Polarization of the incident field

\[ P_{\text{em}} \propto \left| \vec{\mu}_{\text{em}} \cdot \vec{X} \right|^2 \]
- \( \vec{\mu}_{\text{em}} \): Emission dipole moment
- \( \vec{X} \): X polarization detection

Intensity detected by a polarizer in X and Y directions:

\[ I_X(\theta, t) := \left| \mu_{\text{abs}}(\theta) \cdot \vec{E} \right|^2 \left| \mu_{\text{em}}(\theta, t) \cdot \vec{X} \right|^2 \cdot e^{-t/t_f} \]

\[ I_Y(\theta, t) := \left| \mu_{\text{abs}}(\theta) \cdot \vec{E} \right|^2 \left| \mu_{\text{em}}(\theta, t) \cdot \vec{Y} \right|^2 \cdot e^{-t/t_f} \]

- \( t_f \): Molecule fluorescence lifetime.

Anisotropy:

\[ A = \frac{I_X - I_Y}{I_X + I_Y} \]

Function of \( t_{\text{rot}}/t_f, \psi \) and \( \rho \)
Monte Carlo simulations on 1000 molecules

\[ \psi = 130^\circ, \rho = 90^\circ \]

Slow rotational diff. 
\( (\tau_{\text{rot}} \gg \tau_f) \)

Each molecule is fixed in orientation, constraint within the cone

Fast rotational diff. 
\( (\tau_{\text{rot}} \sim \tau_f) \)

Each molecule is free to diffuse within the cone
How to retrieve molecular order information from Single molecule measurements of A
When \( \rho \) is known

The **width** of histogram gives info on rotational diffusion time
The **average** of histo. gives info on rotational constraint:

![Diagram showing anisotropy and rotation angles](image)
Polar dSTORM experimental set-up

- Circular polarization excitation
- Defocus correction in real time, down to 50nm.
- Frame rate: 10-30 ms/image
- Typical number of images for dSTORM reconstruction: 40 000 to 80 000
- A typical localization precision of ~30 nm and probability of false alarm of $1 \times 10^{-6}$
Anisotropy reconstruction

Reconstructed anisotropy image
Polylysine + ATTO655

$A = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$

≈500 molecules analyzed
Vector
Rendering
COS-7 Tubulin--Alexa 647 double antibody

Fluo Image

Super-resolution Image

(full image size 110µm).
Lateral drift Correction

Assumes linear behavior of the lateral drift inside sub-stacks [5]

- A global vector is determined after averaging over all molecules in the image, and kept as a reference for the localization of « couples » and their anisotropy calculation
- Localization precision of a single molecule position is typically 20nm [6]
- Determination precision of A for a single molecule is limited by photon noise
Data analysis single image: Use multiple ρ’s to retrieve information on ψ.
Probing orientational constraint in bio-molecular assemblies
COS-7 Tubulin-Alexa 647 double antibody

Fluo Image

Super-resolution Image

(full image size 110µm).
Tubulin fibers in fixed COS-7 cells

$\psi = 180^\circ$

$< A> \sim 0$

Static disorder
Anisotropy average measured on tubulin fibers (markers with bars :SEM) and simulated (cont. lines) as a function of $\rho$ and $\psi$. 
Actin in vitro

Isotropic due to folded structures?

Visible order $<\psi>_{\text{fiber}} \sim 150^\circ$
Labelled Actin exhibits a degree of molecular order (published value $\psi \sim 100^\circ$)

Anisotropy average of fiber from different images

Anisotropy average measured on single actin fibers (markers with bars: standard error of the mean(SEM)) and simulated (cont. lines) as a function of $\rho$ and $\psi$. 

Actin fibers in vitro
Anisotropy of freely rotating single molecules
The fluorescence polarization anisotropy \( r \) arising from dispersed fluorophores in a liquid medium is closely related to Brownian rotational dynamics of the molecules according to Perrin’s equation:

\[
\frac{1}{r} = \frac{1}{r_0} \left( 1 + \frac{\tau_F}{\tau_R} \right)
\]

where \( \tau_R \) is the rotational correlation time and \( \tau_F \) the fluorescence lifetime.

\[
\tau_R = \frac{V \eta(T)}{k_B T}
\]
**Objective**

**STORM**

- **Gold nanoparticles**
  - size: ~20nm

- **Illumination:**
  - 532 nm
  - 0.1 W

- **CW Laser**
  - 639 nm
  - 4 kW/cm²

- **Dichroic mirror**
- **HWP**
- **Wollaston cube**

- **CCD camera**

- **NA=1.45**

**Heating**
Reconstructed anisotropy image of Atto 633 molecules freely

Anisotropy section map (10x – Pixel size 21.7 nm)
Glycerol Test – STORM
Anisotropy of Atto 633 $1 \times 10^{-10}$ M

- Average anisotropy increases as expected with a higher viscosity
Heating Test – STORM
Anisotropy of Atto 633 1x10^-10 M

Power of laser red = 81 mW, Horizontal excitation, Glycerol percentage = 87 % w/w

Power green = 0 W
Anisotropy Histogram

Counts

Events = 1949
Mean: 0.32
SEM: 0.002

Power green = 0.1 W
Anisotropy Histogram

Counts

Events = 23
Mean: 0.29
SEM: 0.04

SEM = standard error of the mean.
Conclusions

- Some features:
  - Resolution bigger than the diffraction limit.
  - Precision of localization: 20-30 nm
  - Orientation dependence.
  - Single molecules temperature dependence.
  - No invasive approach.
  - Implementation of autofocus.
Future Work - ICFO

SPIM-STORM

10X NA 0.3
Illumination

60X NA 1.0
Illumination

1040 nm 100 fs
Pulsed laser (TPEF)

Emission filter
Wollaston Prism

Hamamatsu
Orca Flash 4.0

488 nm CW laser
(DSTORM)

Flip
Mirror

Axicon

a
b
c
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QUESTIONS?

THANK YOU FOR YOUR ATTENTION…
References: