Advanced optical microscopy: Challenges and prospects

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Live Cell Microscopy

Observation of living cells: Non-Invasive

Light + Far-Field: non-invasive!

Objective → Sample → Far-Field

> 1 μm

Cell diagram:
- microtubules
- mitochondrion
- centriole
- vesicle
- cytosol
- chromatin
- nuclear envelope
- nucleolus
- golgi complex
- lysosome
- flagellum
- plasma membrane
- smooth endoplasmic reticulum
- rough endoplasmic reticulum
- ribosomes
Optical Far-Field Microscopy

Fluorescence

Study specific molecular processes in the living cell:

Fluorescence microscopy
- Label specific protein/molecule

Excite fluorescence by laser light

Liver-Cells: Nucleus and Cell-skeleton
Fluorescence microscopy

- tremendous new insights into biological/cellular processes

- live cell, in-vivo, endogenous, ...
- molecular specificity
- sensitivity (single-molecule)
Far-Field Fluorescence Microscopy

Demands

Biomedical questions have multiple demands

- Temporal resolution
- Spatial resolution
- 3D imaging
- Deep imaging
- Long acquisition times
- Data analysis
T-Cells
Microscopy Demands


RESTING STATE  →  ACTIVATION STATE  →  IMMUNOLOGICAL SYNAPSE

TRIGGERING
Different protein clusters after different points of T-cell/ACP interaction

Yokosuka et al. Nature Immunology 2005

**Challenges:**

Interactions occur

- **On different time scales**
  - Diffusion (µs-ms)
  - Early signaling (ms-s)
  - Microcluster formation (s)
  - Synapse formation (min-h)

- **On different spatial scales**
  - Molecular interactions (nm)
  - Nanoclustering (<200nm)
  - Microclustering (µm)
  - Synapse (>µm)
  - Whole cell (>µm)

**Need a microscope that can cover it all**

Interaction of T-cell with antigen-presenting cell (APC)
Whole gamut of molecules involved!
Optical Microscopy

Trade-Offs

Issue: Need a lot of instruments – sharing/facility

Chemistry:
Label with high photon yield
Optical Microscopy

*Trade-Offs: Solutions*

**Light Sheet Microscope: low phototoxicity**

Lattice light Sheet

- **Lower spatial resolution**
  - 350 nm resolution

**Structured illumination**

- **100 nm resolution**
- TIRF-SIM

**TIRF illumination**

- Low phototoxicity

E. Betzig (Janelia)
Optical Microscopy

*Trade-Offs: Solution – Actin Cytoskeleton*

**Light Sheet Microscope: low phototoxicity**

Lattice light Sheet

- **Lower spatial resolution**
  - 350 nm resolution

**Structured illumination**

- **100 nm resolution**
- **TIRF-SIM**
- **TIRF illumination**
  - Low phototoxicity

**T-cell activation**

**Issues:**

- One layer only
- Resolution still limited

**Improve STED microscopy**

Marco Fritzsche (HIU)
STED Microscopy

Examples - Organelles

⇒ Peroxisomes (protein distribution)

⇒ Data analysis

Silvia Galiani, Dominic Waithe, Katharina Reglinski (HIU)
Lipid Plasma Membrane Organization

Nanoscale

Meso-scale Plasma Membrane Organization:

- Triggers cellular signaling
- Heterogeneous distribution (viscosity, curvature, …)
- Molecular interaction (proteins/lipids)
- Interaction with cortical cytoskeleton

Small spatial scales!!!!
Lipid Plasma Membrane Organization

Interactions on the Nanoscale: Nanodomains

**Lipid rafts/nanodomains?**

- (Transient) cholesterol/sphingolipid-enriched
- Dense molecular packing (ordered)
- Compartmentalize cellular processes

**Problem:**
- heterogeneous
+ highly dynamic
- small (<200 nm)

Missing temporal/spatial resolution → hardly any direct observation method → highly debated

Pike, J.Lipid Res., Keystone meeting 2006
Lingwood/Simons, Science 2010
Lipid Plasma Membrane Organization

Fluorescence Recordings: Lipids

Phosphoglycerolipid:
Atto647N-phosphoethanolamine (PE)

Sphingolipid:
Atto647N-sphingomyelin (SM)

Live PtK2 cells:
physiological conditions
incorporation in plasma membrane
Lipid Plasma Membrane Organization

Imaging of lipid analogs

Homogeneous distribution

Even with STED!

Image acquisition too slow!!!
Lipid Plasma Membrane Dynamics

Measurement mode

Eggeling et al. Nature 2009

Determine average transit times of labeled molecules through observation area

Molecular mobility

Discover interaction/diffusion dynamics!!!
Fluorescence Correlation Spectroscopy (FCS)
Measurement of Diffusion Dynamics

Fluorescence intensity over time

Low fluorescent concentration
⇒ diffusion of single-molecules = fluorescence bursts

Statistics in Time

Fluorescence Correlation Spectroscopy (FCS)
data acquisition - calculation of correlation function
data analysis – length and density of fluctuations

Fitting: anomalous sub-diffusion: \( G(t_c) \sim 1/(1 + (t_c/\tau_d)^\alpha) \)
⇒ transit time \( \tau_d \) (\( \sim \) mass, obs. area) = decay time
\( \sim d^2 / D \)
⇒ anomaly \( 1/\alpha: \)
\( (1/\alpha) = 1: \) normal free diffusion
\( (1/\alpha) > 1: \) anomalous diffusion (e.g. trapping)
Lipid Plasma Membrane Dynamics
Confocal FCS Recordings

Confocal: Limited spatial resolution !!!

Relative large confocal observation area: averages over details on nanoscale cannot distinguish normal diffusion from nanoscale hindered diffusion

SM diffusion slightly prolonged but still normal
$\tau_d \approx 20 \text{ ms (PE)} / 30\text{ms (SM)}$

(1/\alpha) \approx 1 \text{ (PE / SM)}

Slower normal diffusion but no anomalous diffusion???
Lipid Plasma Membrane Dynamics

Confocal FCS Recordings

SM diffusion slightly prolonged but still normal

\[ \tau_d \approx 20 \text{ ms (PE)} / 30\text{ms (SM)} \]

\[ \frac{1}{\alpha} \approx 1 \text{ (PE / SM)} \]

Confocal - FCS

Correlation

Correlation Time [ms]

SM diffusion slightly prolonged but still normal

Slower normal diffusion

but no anomalous diffusion???
Far-Field Microscopy
*Surpassing the Resolution Limit: Turning ON/OFF*

\[ \Delta x = \frac{\lambda}{2n \sin \alpha} \]

Ernst Abbe 1873

Modulate fluorescence
Turn OFF all objects but at a single point

STED

Observation area
Resolution << 200 nm

Laser → Lens → Detector

axial \sim 600 \text{ nm}
lateral \sim 200 \text{ nm}
Far-Field Microscopy

Surpassing the Resolution Limit: Turning ON/OFF

Ernst Abbe 1873

Laser

axial ~ 600 nm

Lens

Detector

lateral ~ 200 nm

⇒ Inhibit fluorescence (reversibly)

Stimulated Emission

\[ S_0 \leftrightarrow S_1 \]

Excitation

Fluorescence

\[ \tau_{\text{vib}} \approx 1\text{ ns} \]

Stimulated Emission

\[ \tau_{\text{sub}} \approx 1\text{ ps} \]
Lipid Plasma Membrane Dynamics

*Move to STED*

Confocal: Limited spatial resolution !!!

STED!!!!
Lipid Plasma Membrane Dynamics

Move to STED

STED (240 -> 40nm):
PE diffusion scales with area reduction
\[ \tau_d: 20 \rightarrow 0.6 \text{ms (35-fold)} \]
and still normal
\[(1/\alpha) \approx 1 \]

STED:
SM diffusion much longer than PE
\[ \tau_d: 30 \rightarrow 3 \text{ms (10-fold)} \]
and anomalous
\[(1/\alpha) \approx 1.5 \]
STED Microscopy

Dynamical confinement of resolution

Nanoscale observation areas: CONTINUOUS TUNING of spatial resolution!
Lipid Plasma Membrane Dynamics

**STED-FCS: Measurement Principle**

**STED-FCS**

Determine transit time for different sizes of observation areas (different STED intensities)

Calculate apparent diffusion coefficient:

\[ D \sim \text{area} / \text{transit time} \]

Dependencies: \( D(\text{diameter}) \)

240nm → 30/40nm

Varies for different diffusion modes
Lipid Plasma Membrane Dynamics

**STED-FCS: Diffusion modes**

Free diffusion

- Wawrezinieck et al. *Biophys J.* 2005 December; 89(6)
- Mueller et al. *Biophys J* 2011

![STED Intensity](image)

Apparent Diffusion Coefficient $[\mu m^2/s]$

Observation Diameter $[nm]$

$D \sim \text{area} / \text{transit time}$
Lipid Plasma Membrane Dynamics

STED-FCS: Diffusion modes

Free diffusion

STED Intensity

Wawrezinieck et al. Biophys J. 2005 December; 89(6)
Eggeling et al. Nature 457, 1159-1162, 2009
Mueller et al. Biophys J 2011

Apparent diffusion coefficient:

\[ D \sim \frac{\text{area}}{\text{transit time}} \]
Lipid Plasma Membrane Dynamics

**STED-FCS: Diffusion modes**

**Free diffusion**

Apparent diffusion coefficient: 

\[ D \sim \frac{\text{area}}{\text{transit time}} \]

Wawrezinieck et al. Biophys J. 2005 December; 89(6)


Mueller et al. *Biophys J* 2011

Trapping

Domain incorporation

Apparent diffusion coefficient:
Lipid Plasma Membrane Dynamics

*STED-FCS: PE vs SM*

PE: free diffusion (weak trapping)

SM: trapping

→ Complex on molecular scale
   (proteins, lipid-shells, …)

~10 ms, no movement during trapping

Cholesterol-assisted
   (COase/β-Cyclo-Dextrin/Zaragozic acid…)

Binding partner bound to cytoskeleton
   (Latrunculin/Jasplakinolide/Nocodazole…)

Lipid specific (but not dye-label)
   (variation of lipid structure, dye/position)

Mueller et al. *Biophys J* 2011
Lipid Plasma Membrane Dynamics

**STED-FCS: PE vs SM**

**Message**
- STED allows combination with FCS
- Take advantage of tuning of observation spot
- STED-FCS can highlight molecular interactions
- Lipid-specific interactions in plasma membrane

**But:**
No sign of a nanodomain incorporation
STED Microscopy

Photobleaching/Phototoxicity

Simultaneous excitation with Excitation and STED light

Two effects:

⇒ Shortening of excited state lifetime
⇒ Reduction of photobleaching

⇒ Excited state absorption - photobleaching

Eggeling et al., QuartRevBiophys 2015
STED Microscopy
Photobleaching/Phototoxicity

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Eggeling et al, Quart Rev Biophys 2015
STED Microscopy
Photobleaching/Phototoxicity

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⇒ Reduction of photobleaching

⇒ Excited state absorption - photobleaching

Issue:
- Find appropriate dye
- Optimize laser wavelength

Eggeling et al, QuartRevBiophys 2015
Photobleaching

⇒ minimize triplet population

Donnert et al, PNAS 2006; Donnert et al, Nat. Meth. 2007; Donnert et al, Photochem Photobiol 2009)
STED Microscopy
Photobleaching/Phototoxicity

Photobleaching
⇒ minimize triplet population

⇒ Fast scanning

⇒ relaxation of triplet
+ less triplet = more signal

Donnert et al, PNAS 2006; Donnert et al, Nat. Meth. 2007; Donnert et al, Photochem Photobiol 2009)
Optimization of Fluorescence Signal

**STED – D-Rex**

**Issue:**
- Optimize setup
- Optimize laser

**Slow Scanning**

- $\frac{1}{\Delta T} = 80 \text{ MHz}$
- $\Delta T < \tau_D$
- Bleach

**Fast Scanning**

- $\frac{1}{\Delta T} = 250 \text{ kHz}$
- $\Delta T > \tau_D$
- No Bleach
STED Microscopy

Ultimate limit: NV centers in diamond

Nitrogen vacancies (NV) centers in diamond

Ultrastable luminescence sources (Gruber et al. Science 1996)

Excitation 532nm – Luminescence ~700nm
STED Microscopy
Single NV centers – ultimate photostablility

STED on “isolated“ NV centers
in diamond of type IIa grown by chemical vapour deposition (Jelezko, Wrachtrup (Stuttgart))

exc: 532nm - STED: 775nm 8MHz

Ultrastable: apply very high STED intensities

$\Delta r \approx \frac{\lambda}{2n \sin \alpha \sqrt{1 + I / I_{sat}}}$

$\propto \frac{\lambda_{STED}}{\sqrt{I_{max,STED} / I_s}}$

$I_{STED} = 3.5 \text{ GW/cm}^2$

Fluorescence Nanoscopy

**GSD imaging by optical dark state shelving**

Fluorescence of NVs can be photoswitched via dark state

**GSD: Ground-State-Depletion**
Fluorescence Nanoscopy

GSD imaging by optical dark state shelving
Far-Field RESOLFT Nanoscopy
Reversibly Photoswitchable Fluorescent Proteins

Photoswitchable Proteins

Dronpa → rsFastLime
Stiel et al, Biochem J 2007

GFP → photoswitching (rsEGFP)
Grotjohann et al, Nature 2011

Switch-off + Readout: 488nm
Switch-on: 405nm

Photoisomerisation
cis-trans conformational states
dark (trans)- bright (cis)
Andresen et al. (2005) PNAS

ON/OFF at low CW powers
nW - µW (~ kW/cm²)

ON-Light + Readout-Light  +  OFF-Light  →  Sub-diffraction

RESOLFT = Reversible Saturable Optical Fluorescence Transition
Far-Field RESOLFT Nanoscopy
Reversibly Photoswitchable Fluorescent Proteins

GFP → photoswitching (rsEGFP)
Grotjohann et al, Nature 2011

Switch-off + Readout: 488nm
Switch-on: 405nm

E. coli bacterium expressing rsEGFP–MreB
bacterial actin homologue MreB

Low intensity ≈ 1 kW/cm²

Excellent for Live-Cell (low light levels)

Issue:
Photoswitchable proteins / dyes

Scanning = slow
Far-Field RESOLFT Nanoscopy

Reversibly Photoswitchable Fluorescent Proteins

Thousand doughnuts (CCD detection)

Chmyrov et al, Nature Meth. 2013
Far-Field RESOLFT Nanoscopy
Reversibly Photoswitchable Fluorescent Proteins

Parallelization

Intensity $\approx 1 \text{ kW/cm}^2$

Keratin19-rsEGFP expressed in living PtK2 cells

Scale 10$\mu$m

$120 \times 100 \mu m - 1 s$
Chmyrov et al, Nature Meth. 2013
Combination of fluorescence readout:
- Force
- Structure
- Electrophysiology (currents)
- ...

Trade-offs: Complementary Techniques
Advanced Microscopy

Trade-offs: Complementary Techniques

Traction-Force Microscopy
Advanced Microscopy

Trade-offs: Complementary Techniques

**Traction-Force Microscopy**

Diagram showing a cell on a functionalized surface with fluorescent beads embedded in an elastic gel on a glass slide.
Advanced Microscopy

Trade-offs: Complementary Techniques

Traction-Force Microscopy
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