Single Molecules, Cells, and Super-Resolution Optics

Eric Betzig
Janelia Research Campus, HHMI
Cornell and the Beginnings of Near-Field Optical Microscopy

Mike Isaacson and his STEM

Me, Alec Harootunian, and Aaron Lewis, 1983

concept

The Long History of Breaking Abbe’s Law: Near-Field

Sir Eric Ash

Edward “Hutchie” Synge, *Phil. Mag.* 6, 356 (1928)

- J.A. O’Keefe (1956)
- A.V. Baez (acoustics, 1956)
- C.W. McCutchen (1967)
- U. Ch. Fischer (lithography, 1981)
- D.W. Pohl (1984)
- J. Wessel (1985)

near-field microwave ($\lambda = 3$ cm) microscopy

Resolution of 1/60 of the wavelength!

The Long History of Breaking Abbe’s Law: Far-Field

Structured Light

Optical Systems with Resolving Powers Exceeding the Classical Limit

W. Lukosz
Institut A für Physik, Technische Hochschule, 33 Braunschweig, Germany
(Received 27 April 1966)

W. Lukosz, JOSA 56, 1463 (1966)

Nonlinear Interaction with Sample

integrated circuit linewidth control

A Priori Information: wafer inspection

Sir Eric Ash

Resolution 3× beyond Abbe’s Limit!

A. Bachl, W. Lukosz, JOSA 57, 163 (1967)
Making Near-field *Optical* Microscopy Work

Edwin Neher and Bert Sakmann, Nobel 1991

Me, Alec Harootunian, and Aaron Lewis, 1983

patch clamp: single ion channel recording


end of aluminum coated pipette

50 nm aperture
Making Near-field *Optical* Microscopy Work

my near-field scanning optical microscope (NSOM)

microscope control room

diffraction limited

NSOM

NSOM

NSOM

NSOM
Initial Struggles at Bell Labs

AT&T Bell Labs, Murray Hill, NJ

Horst Störmer, 1998 Nobel in Physics

retroreflection in pipette

lowest order waveguide modes at tip
Making NSOM Routine

adiabatically tapered optical fiber probe

shear force distance regulation


The Golden Age of NSOM

high density data storage

photolithography


histological section, monkey hippocampus

fluorescence: phase change in phospholipid monolayers


nanoscale spectroscopic imaging
Single Molecule Detection (SMD)

fluence: actin, mouse fibroblast cell
widefield NSOM


Nobel, 2014

W.E. Moerner


SM fluorescence excitation spectrum, 1.8°K

Michel Orrit


single molecule absorption spectra, 1.6°K

W.E. Moerner


Time gated:


FCS:
NSOM and the Birth of Single Molecule Microscopy

Rob Chichester

E. Betzig, R.J. Chichester, Science 262, 1422 (1993)

Horst Störmer
NSOM and the Birth of Single Molecule Microscopy

single molecule fluorescence anisotropy

random

dil-C_{18}-(3) molecules on PMMA

E. Betzig, R.J. Chichester, Science 262, 1422 (1993)

Hans Bethe, 1967 Nobel in Physics

H.A. Bethe, Phys. Rev. 66, 163 (1944)

E fields at aperture: theory vs. experiment

\[
\begin{align*}
|E_x|^2 & \quad |E_y|^2 \\
|E_z|^2 &
\end{align*}
\]

\[z/a = 0.1 \quad z/a = 0.2 \quad \text{data} \quad z/a = 0.4 \quad z/a = 0.8\]
NSOM and the Birth of Single Molecule Microscopy

- first imaging of single molecules at room temp
- first super-resolution imaging of single molecules
- first measurement of single molecule dipole orientations
- first localization of single molecules to fraction of PSF width (12 nm xy, 6 nm z)

Rob Chichester

Cryogenic Near-field Spectroscopy

scanning tunnel spectroscopy of Abrikosov flux lattice in NbSe$_2$


Alexei Abrikosov, 2003 Nobel in Physics
Cryogenic Near-field Spectroscopy

Harald Hess

Harald’s low temp STM

NSOM fiber probe

GaAs / AlGaAs multiple quantum well

Alferov & Kroemer, 2000 Nobel in Physics

semiconductor laser diode
Cryogenic Near-field Spectroscopy

single exciton transitions, 23Å quantum well, 2°K

exciton energy variations due to interface roughness

exciton recombination sites scrolling from $\lambda = 700$ to $\lambda = 730$ nm

isolation of discrete sites in $x, y, \lambda$ space

My First Mid-Life Crisis

NSOM engineering limitations:

- poor yield during manufacture
- fragile probes
- topographical artifacts
- weak signals
- probe tips get hot
- large probe tip (0.25 μm)

Cells aren’t flat!

NSOM fundamental limitations:

- probe perturbs fields at sample
- complex contrast mechanisms
- nonlinear image formation - artifacts
- the near-field is VERY, VERY short


My First Mid-Life Crisis

me and Harald, 1989

me and Harald, 1994

NSOM fundamental limitations:

- probe perturbs fields at sample
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- the near-field is very, very short

Multidimensional Localization Microscopy


Spatial Resolution and the Nyquist Criterion

Nyquist criterion:
Sampling interval must be at least twice as fine as the desired resolution

20 samples / period

2 samples / period

<table>
<thead>
<tr>
<th>Dimensionality</th>
<th>Molecules Required per Diffraction Limited Region for 20 nm Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D</td>
<td>25</td>
</tr>
<tr>
<td>2D</td>
<td>500</td>
</tr>
<tr>
<td>3D</td>
<td>$2.9 \times 10^4$</td>
</tr>
</tbody>
</table>

Diffraction Limited Region: 0.25 μm dia, 0.6 μm long
And Now for Something Completely Different

Flexible Adaptive Servohydraulic Technology (FAST)

- moves 4000 kg load at 8g acceleration
- positioning precision to 5 µm

Robert Betzig
My Second Mid-Life Crisis
Searching for a New Direction

me in Joshua Tree National Park

me in Oahu, Hawaii

Harald in Sedona, Arizona

Harald in Yosemite National Park
Fluorescent Proteins Revolutionize Biological Imaging

Shimomura, Chalfie, & Tsien

1994: green fluorescent protein

2008: Chemistry Nobel

microtubule ends

dendoplasmic reticulum

golgi (green), mitochondria (red)
Switching Behavior in Green Fluorescent Protein

488 nm absorption increase under 398 nm illumination

proposed mechanism


W.E. Moerner, 2014 Nobel in Chemistry

photoactivation energy diagram

Directed Mutagenesis of Photoactivated Fluorescent Proteins (PA-FPs)

increased on/off contrast of PA-GFP

Jennifer Lippincott-Schwartz

George Patterson

pulse chase: nuclear vs cytosolic diffusion

A Fateful Trip

Greg Boebinger
National High Magnetic Field Lab

Mike Davidson
Neckties®

Tallahassee, Florida

website tutorials

Zeiss

Olympus

Nikon
Finding the Missing Link

La Jolla Labs
Assembling the Rest of the Team

Jennifer Lippincott-Schwartz

George Patterson

the microscope in the darkroom in Jennifer’s lab
Photoactivated Localization Microscopy (PALM)

lysosomes, COS-7 cell, Kaede-tagged CD63

single molecule frames  integrated image  PALM image

~80 nm cryosection:  • low autofluorescence
                    • immobile PA-FPs
                    • image internal organelles

Photoactivated Localization Microscopy (PALM)

lysosomes, COS-7 cell, Kaede-tagged CD63

A High On/Off Contrast Ratio is Essential for High Resolution

paxillin, focal adhesions

EosFP > 2000:1  PA-GFP < 75:1

diffraction limited TIRF

Eos FP and caged Q-rhodamine support Nyquist-defined sub-20 nm resolution

caged Q-rhodamine, > 1000:1

time

diffraction limited TIRF

From Rags to Riches, Thanks to HHMI

Janelia Research Campus

The Boss: Gerry Rubin

Endless Coffee

Hari Shroff

my PALM

Gleb Shtengel

Harald’s iPALM
PALM Application Examples

Chemotaxis Receptors in *E. coli* 

Actin Polymerization in Dendritic Spines 

Two-Color Imaging of Focal Adhesion Proteins 

Regulation of Gene Expression During Myogenesis 
iPALM: Ultrasensitive PALM in 3D

Harald Hess

iPALM schematic

single focal adhesion

iPALM xz view

vertical architecture of adhesions


three phase single molecule interferometry

ESCRT machinery at HIV budding sites

Correlative Electron Microscopy and PALM

first correlative EM with super-resolution: mitochondria


3D correlative EM/PALM

mitochondria (B&W – FIB SEM)
mitochondrial DNA (red - iPALM)


scrolling plane-by-plane thru 3D

B.G. Kopek, et al., PNAS, 109, 6136 (2012)

cell membrane (B&W - TEM) & clathrin (color - iPALM)

Overlaid iPALM – TEM

3D TEM tomogram

0.5 micron
Caveats with Super-Resolution Microscopy: Fixed Cells

extremely high labeling densities required

initial density 4x higher density

exogenous dyes: limited affinity & high background

overexpression of protein

overexpressed

physiologically expressed

fixation artifacts, endoplasmic reticulum

live cell fixed
Particle Averaging Improves Resolution of Stereotypic Structures

nuclear pore complex proteins

positions determined to < 1 nm

### Nyquist criterion:

\[ N \text{-fold resolution increase in } D \text{ dimensions} \rightarrow N^D \text{-fold more photons collected} \]

<table>
<thead>
<tr>
<th>Technique</th>
<th>L. Schermelleh, R. Heintzmann, <em>J. Cell Biol.</em> (2010)</th>
<th>reported resolution (nm)</th>
<th>photon increase required</th>
<th>intensity (W/cm²)</th>
<th>acquisition time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STED / RESOLFT</td>
<td>Scanning PMT/APD + STED pulse PSF = Effective PSF (PSF shaping)</td>
<td>xy: 20 nm</td>
<td>100</td>
<td>[10^4 - 10^9]</td>
<td>&gt; 60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>xyz: 30 nm</td>
<td>1,070</td>
<td></td>
<td>~1,000</td>
</tr>
<tr>
<td>Localization</td>
<td>Wide-field CCD</td>
<td>xy: 20 nm</td>
<td>100</td>
<td>[10^3 - 10^4]</td>
<td>&gt;20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>xy: 10 nm, z: 20 nm</td>
<td>14,400</td>
<td></td>
<td>1,500</td>
</tr>
<tr>
<td>SIM</td>
<td>Wide-field CCD + 5 phase shifts + Interference of exciting light with sample structure (Moiré effect) = Mathematic reconstruction</td>
<td>xy: 100 nm</td>
<td>4</td>
<td>[10 - 10^2]</td>
<td>0.1 - 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>xy: 100 nm, z: 370 nm</td>
<td>8</td>
<td></td>
<td>~10</td>
</tr>
</tbody>
</table>
Live Cell Structured Microscopy

endoplasmic reticulum

2D SIM, 98 nm resolution
0.1 sec acquisition, 1800 frames

cloathrin coated pits and cortical actin

TIRF-SIM, 82 nm resolution
0.5 sec acquisition, 90 frames

early endosomes and cortical actin

Nonlinear SIM, 62 nm resolution
1.5 sec acquisition, 34 frames
The Challenges and Importance of Studying Live Cell Dynamics

tradeoffs, tradeoffs, tradeoffs

spatial resolution

phototoxicity

temporal resolution

imaging depth

Life is Animate

dividing HeLa cell

prometaphase

velocity/track (μm/s)
Lattice Light Sheet Microscopy: Non-Invasive 4D Live Cell Imaging

Concept

T cell and its target cell

Chromosomes, mitos, and ER during mitosis

Tetrahymena thermophila

C. elegans early embryo

Bi-Chang Chen, Wes Legant, Kai Wang

Ultra-High Density 3D Localization Microscopy

Points Accumulation for Imaging in Nanoscale Topography (PAINT)

Widefield vs. PAINT

A. Sharonov, R.M. Hochstrasser, PNAS 103, 18911 (2006)

3D PAINT with lattice: dividing cell

intracellular membranes, COS-7 cell

over 300 million localized molecules
Adaptive Optics (AO): Moving Cell Biology Away from the Cover Slip

non-scattering media: zebrafish embryonic brain

Kai Wang

scattering media: mouse visual cortex

Na Ji

dendritic spines, 600 μm deep

AO off

AO on

5 μm

functional imaging of neural activity, 400 μm deep

AO OFF

AO ON
The Beauty and Complexity of Living Systems