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

A. Lewis, et al.,  
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

object

image

Resolution of 1/60 of the wavelength!

E.A. Ash, G. Nicholls, Nature 237, 510 (1972)
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

nominal exposure

intentional overexposure

A Priori Information: wafer inspection

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

Making Near-field *Optical* Microscopy Work

my near-field scanning optical microscope (NSOM)

microscope control room

diffraction limited

NSOM

NSOM
Initial Struggles at Bell Labs

Horst Störmer, 1998 Nobel in Physics
Making NSOM Routine

adiabatically tapered optical fiber probe

shear force distance regulation


The Golden Age of NSOM

high density data storage

photolithography

fluorescence: phase change in phospholipid monolayers


nanoscale spectroscopic imaging

histological section, monkey hippocampus


Single Molecule Detection (SMD)

fluorescence: actin, mouse fibroblast cell
widefield
NSOM

E. Betzig, et al., Bioimaging 1, 129 (1993)

Nobel, 2014
W.E. Moerner


SM fluorescence excitation spectrum, 1.8°K

Time gated:

FCS:
R. Rigler, J. Widengren, Bioscience 3, 180 (1990)


Michel Orrit

SM fluorescence bursts at room temp

100 fm R6G IN WATER

WATER

5 GHz

0.05 GHz

EXCITATION FREQUENCY

FLUORESCENCE INTENSITY

0.5 GHz

TIME (s)
NSOM and the Birth of Single Molecule Microscopy

single molecule fluorescence anisotropy
random

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


Horst Störmer

single molecule NSOM signal $|E(x) \cdot p|^2$
NSOM and the Birth of Single Molecule Microscopy

Rob Chichester

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

\[ |E_x|^2 \]
\[ |E_y|^2 \]
\[ |E_z|^2 \]

data

z / a = 0.1  z / a = 0.2  z / a = 0.4  z / a = 0.8

200 nm
NSOM and the Birth of Single Molecule Microscopy

- Single molecule fluorescence anisotropy
  - Random orientation

- 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)

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

Alferov & Kroemer, 2000 Nobel in Physics

GaAs / AlGaAs multiple quantum well

semiconductor laser diode

NSOM fiber probe
Cryogenic Near-field Spectroscopy

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

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

exciton energy variations due to interface roughness

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

NSOM fundamental limitations:

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- complex contrast mechanisms
- nonlinear image formation - artifacts

- the near-field is very, very short

me and Harald, 1989

me and Harald, 1994

Multidimensional Localization Microscopy

Proposed method for molecular optical imaging

E. Betzig

NSOM Enterprises, 17 Webster Drive, Berkeley Heights, New Jersey 07922

Photobleaching:

Lifetime:

Blinking:

A.M. van Oijen, et al., JOSA A16, 909 (1999)

spectral isolation

higher dimensional isolation

original image

localization

1 μm

Axial direction

Lateral plane
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

endoplasmic reticulum

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

488 nm absorption increase under 398 nm illumination


W.E. Moerner, 2014 Nobel in Chemistry

in vivo UV photoactivation (PA) of wtGFP

before PA  after PA


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®

website tutorials

Zeiss

Olympus

Nikon

Tallahassee, Florida
Finding the Missing Link

Assembling the Rest of the Team

Jennifer Lippincott-Schwartz
George Patterson

the microscope in the darkroom in Jennifer’s lab

Rob Tycko, NIDDK
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

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

caged Q-rhodamine, > 1000:1

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


Hari Shroff
iPALM: Ultrasensitive PALM in 3D

Harald Hess

single focal adhesion

vertical architecture of adhesions


iPALM schematic

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)

K. Sochaki, et. al, Nat. Methods, 11, 305 (2014)

3D TEM tomogram

scrolling plane-by-plane thru 3D


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

cell membrane (B&W - TEM) & clathrin (color - iPalm)
Caveats with Super-Resolution Microscopy: Fixed Cells

extremely high labeling densities required

initial density  4x higher density

overexpression of protein

overexpressed  physiologically expressed

exogenous dyes: limited affinity & high background

fixation artifacts, endoplasmic reticulum
Particle Averaging Improves Resolution of Stereotypic Structures

Nup107-160 subcomplex

positions determined to < 1 nm

### Localization

<table>
<thead>
<tr>
<th>Technique</th>
<th>Nyquist Criterion</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>$N$-fold resolution increase in $D$ dimensions $\rightarrow N^D$-fold more photons collected</td>
<td>xy: 20 nm, xyz: 30 nm</td>
<td>1,070</td>
<td>$10^4 - 10^9$</td>
<td>&gt; 60</td>
</tr>
<tr>
<td>Localization</td>
<td></td>
<td>xy: 20 nm</td>
<td>100</td>
<td>$10^3 - 10^4$</td>
<td>&gt;20</td>
</tr>
<tr>
<td>SIM</td>
<td></td>
<td>xy: 100 nm, z: 20 nm</td>
<td>14,400</td>
<td></td>
<td>1,500</td>
</tr>
<tr>
<td>SIM</td>
<td></td>
<td>xy: 100 nm</td>
<td>4</td>
<td>$10 - 10^2$</td>
<td>0.1 - 1</td>
</tr>
<tr>
<td>SIM</td>
<td></td>
<td>xy: 100 nm, z: 370 nm</td>
<td>8</td>
<td></td>
<td>~10</td>
</tr>
</tbody>
</table>

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**Caveats with Super-Resolution Microscopy: Live Cells**

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**Nyquist criterion:**

$N$-fold resolution increase in $D$ dimensions $\rightarrow N^D$-fold more photons collected
Live Cell Structured Microscopy

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

clathrin 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

Mats Gustafsson, 1960-2011

Dong Li
Lin Shao
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
Lattice Light Sheet Microscopy: Non-Invasive 4D Live Cell Imaging

T cell and its target cell

concept

chromosomes, mitos, and ER during mitosis

Tetrahymena thermophila

C. elegans early embryo

Ultra-High Density 3D Localization Microscopy

Points Accumulation for Imaging in Nanoscale Topography (PAINT)

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

3D PAINT with lattice: dividing 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