From single cell
to single molecule analysis

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Typical eucaryotic somatic cell

Size 5 - 10 μm
Volume ~ 500 fl
Total mass ~ 500 pg

Proteins:

Only 10% of the cellular mass form about 2 fmol of 10 000 expressed proteins

A typical mammalian cell contains from 100 to 500 pg of protein.

Cellular receptors > 1000 molecules; 60 ms life-time of complex molecule entering cell - receptor

2000 glycans per cell

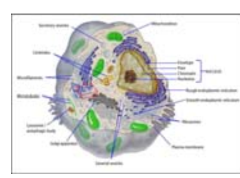
Various signaling enzymes $10^3 – 10^5$ molecules

Structural proteins 10^8 molecules

DNA:

nucleus: 20 % of cell mass

DNA ~5 pg (MCF 7 cells)



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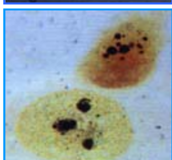
Cells as Test Tubes

Single cell or single molecule analyses provide:

- space – time correlation of cellular organization and dynamics
- no ensemble averaging
- absolute sensitivity – no separation needed
- transition paths observable only in single cell/molecule
- distribution of behaviors – static or dynamic heterogeneity. Tumor heterogeneity is one of the major causes of cancer drug resistance.



Important molecules - analytes:

- at maximum concentrations
- already separated
- at biologically relevant positions

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Methods of single cell analysis

Imaging methods
- Optical microscopies (far-, near-field)
- Correlative light and electron microscopy
- Mass spectrometry imaging

Detection methods
- Laser induced fluorescence (LIF)
- Total internal reflection fluorescence (TIRF)
- Surface enhanced Raman scattering (SERS)
- Nonlinear optical spectroscopy
- Foerster resonance energy transfer (FRET)
- Chemi- and bioluminescence detection

(Imaging and detection methods: nm, ps resolution, absolute sensitivity)

Separation methods
HPLC, CE, IEF, on-line multidimensional separations with MS detection and identification

(Separation methods: hundreds zmol inj., tens pM conc.)

low throughput (imaging methods)

high throughput (detection and separation methods)

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Mass Spectrometry

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Mass Spectrometry Imaging

Matrix assisted laser desorption and ionization (MALDI)

Secondary ion mass spectrometry (SIMS)

MS imaging spatial resolution <1 μm at ambient pressures, with very high mass accuracy and mass resolution.

Subcellular resolution for many kinds of cells.

Spatial resolution 5 - 30 μm - for generating enough signal per spot

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Single cell proteomics

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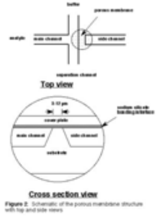
J. Michael Ramsey

Minnie N. Goldby Distinguished Professor of Chemistry

Department of Chemistry
The University of North Carolina at Chapel Hill
Chapel Hill, NC USA



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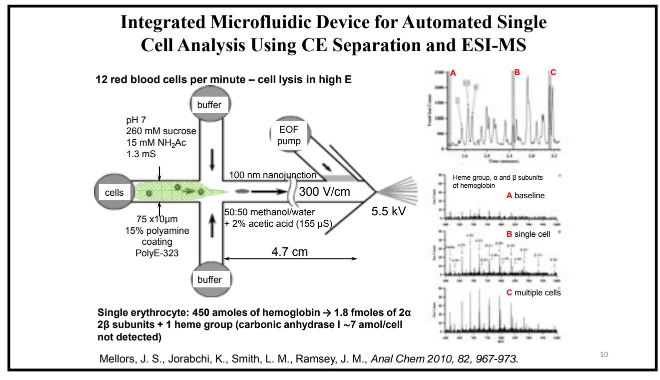
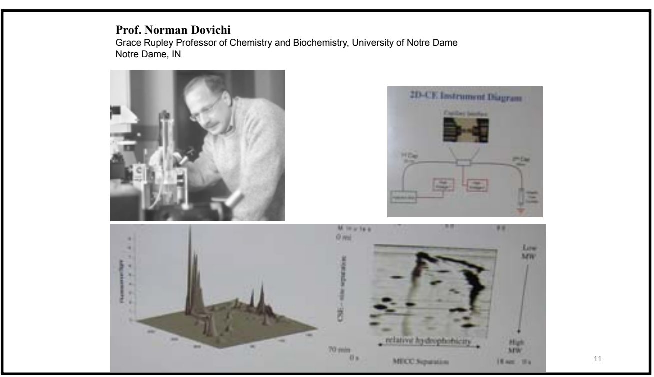
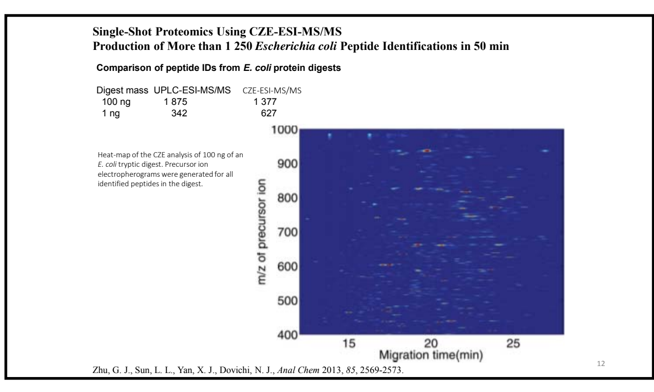

Integrated Microfluidic Device for Automated Single Cell Analysis Using CE Separation and ESI-MS
12 red blood cells per minute – cell lysis in high E
buffer
pH 7
260 mM sucrose
15 mM NH2Ac
1.3 mS
EOF
pump
100 nm nanojunction
cells
300 V/cm
5.5 kV
75 x10µm
15% polyamine
coating
PolyE-323
50:50 methanol/water
+ 2% acetic acid (155 µS)
4.7 cm
buffer
A
B
C
Heme group, α and β subunits
of hemoglobin
A baseline
B single cell
C multiple cells
Single erythrocyte: 450 amoles of hemoglobin → 1.8 fmoles of 2α 2β subunits + 1 heme group (carbonic anhydrase I ~7 amol/cell not detected)
Mellors, J. S., Jorabchi, K., Smith, L. M., Ramsey, J. M., Anal Chem 2010, 82, 967-973.
10


Prof. Norman Dovichi
Grace Rupley Professor of Chemistry and Biochemistry, University of Notre Dame
Notre Dame, IN
2D-CE Instrument Diagram
relative hydrophobicity
MECC Separation
Low
MW
High
MW
11


Single-Shot Proteomics Using CZE-ESI-MS/MS
Production of More than 1 250 Escherichia coli Peptide Identifications in 50 min
Comparison of peptide IDs from E. coli protein digests
Digest mass UPLC-ESI-MS/MS CZE-ESI-MS/MS
100 ng 1 875 1 377
1 ng 342 627
Heat-map of the CZE analysis of 100 ng of an E. coli tryptic digest. Precursor ion electropherograms were generated for all identified peptides in the digest.
m/z of precursor ion
1000
900
800
700
600
500
400
15
20
25
Migration time(min)
Zhu, G. J., Sun, L. L., Yan, X. J., Dovichi, N. J., Anal Chem 2013, 85, 2569-2573.
12

2D CE-MS system with immobilized enzyme reactor

1st capillary:
separation of protein mixture for 8 min

Replaceable enzymatic microreactor (trypsin immobilized on magnetic particles captured by a pair of narrow magnets at the capillary outlet) on-line protein digestion in the reactor during the separation of peptides for 1 min

2nd capillary:
portions of created peptides are periodically introduced via the first interface into the second capillary for separation

Sheath-flow electrospray
transfer of peptides via the second interface into a emitter.

2D separation requires roughly 30min to digest and separate 30 fractions.

Li, Y. H., Wojcik, R., Dovichi, N. J., *J. Chromatogr. A* 2011, *1218*, 2007–2011.

A: Protein separation capillary; Flush; Interface 1; Magnets; Peptide separation capillary; Interface 2; Flush; Electrospray emitter; HV supply 1; HV supply 2; HV supply 3; Protein separation buffer; Peptide separation buffer; Electrospray sheath buffer

B: insulin chain b oxidized; beta-casein; Scan number; Analysis time (min); m/z

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Integrated CZE−ESI-MS/MS System with an Immobilized Trypsin Microreactor for Online Digestion and Analysis of Picogram Amounts of RAW 264.7 Cell Lysate

Triplicate analysis of RAW 264.7 cell lysates:

3 ng 7 ± 2 protein groups identified

300 pg 2 ± 1 protein groups identified

(relatively highly abundant proteins)
MM: 5.7 - 58.8 kDa; pI: 4.5 - 11.0

Protein amounts analyzed correspond to the protein content in three cells

Electrokinetically pumped sheath-flow cuvette nanospray interface, i.d. 8 µm

Microreactor-CZE-ESI-MS/MS

Microreactor; Separation capillary; Flush; Interface; LTQ-Orbitrap Velos; MS; Separation buffer; HV Supply I; 50% (v/v) metOH 0.05% (v/v) FA; Sheath flow buffer; HV Supply II

Uncoated capillary: 34 cm, 2 cm reactor, 3 min digestion time, Buffer: 5 mM NH_4HCO_3 (pH 8.0)

Triplicate extracted ion electropherograms of PMFIVNTNVPR peptide from the macrophage migration inhibitory factor

Sun, L. L., Zhu, G. J., Dovichi, N. J., *Anal Chem* 2013, *85*, 4187-4194.

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Single cell metabolomics

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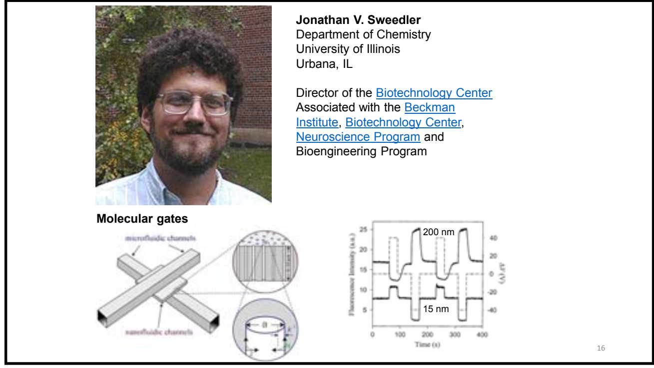
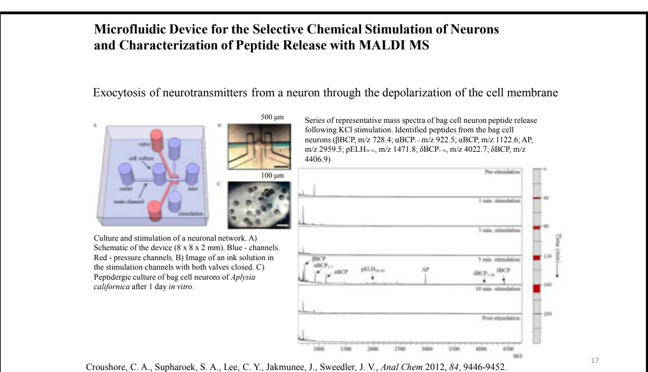
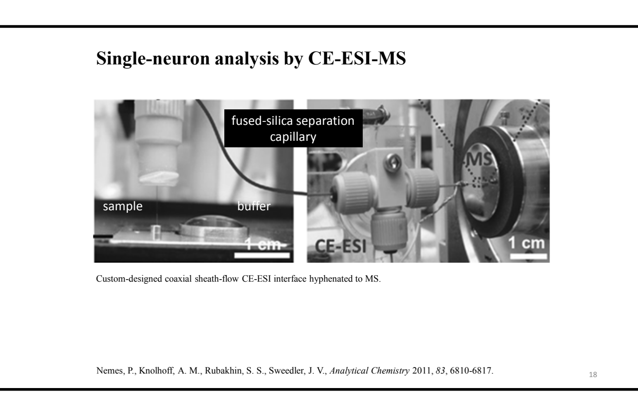


Jonathan V. Sweedler
Department of Chemistry
University of Illinois
Urbana, IL
Director of the Biotechnology Center
Associated with the Beckman Institute, Biotechnology Center, Neuroscience Program and Bioengineering Program
Molecular gates
200 nm
15 nm
Time (s)
16



Microfluidic Device for the Selective Chemical Stimulation of Neurons and Characterization of Peptide Release with MALDI MS
Exocytosis of neurotransmitters from a neuron through the depolarization of the cell membrane
500 µm
100 µm
Series of representative mass spectra of bag cell neuron peptide release following KCl stimulation. Identified peptides from the bag cell neurons (βBCP, m/z 728.4; αBCP1-7 m/z 922.5; αBCP, m/z 1122.6; AP, m/z 2959.5; pELH30-43, m/z 1471.8; δBCP1-36, m/z 4022.7; δBCP, m/z 4406.9)
Culture and stimulation of a neuronal network. A) Schematic of the device (8 x 8 x 2 mm). Blue - channels. Red - pressure channels. B) Image of an ink solution in the stimulation channels with both valves closed. C) Peptidergic culture of bag cell neurons of Aplysia californica after 1 day in vitro.
Croushore, C. A., Supharoek, S. A., Lee, C. Y., Jakmunee, J., Sweedler, J. V., Anal Chem 2012, 84, 9446-9452.
17



Single-neuron analysis by CE-ESI-MS
fused-silica separation capillary
sample
buffer
1 cm
CE-ESI
MS
1 cm
Custom-designed coaxial sheath-flow CE-ESI interface hyphenated to MS.
Nemes, P., Knolhoff, A. M., Rubakhin, S. S., Sweedler, J. V., Analytical Chemistry 2011, 83, 6810-6817.
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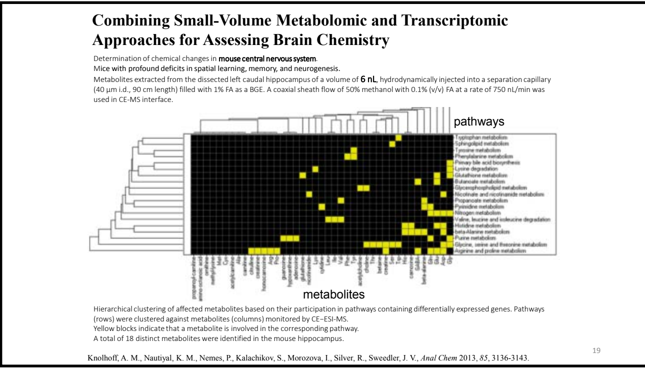
Combining Small-Volume Metabolomic and Transcriptomic Approaches for Assessing Brain Chemistry

Determination of chemical changes in **mouse central nervous system**.
Mice with profound deficits in spatial learning, memory, and neurogenesis.
Metabolites extracted from the dissected left caudal hippocampus of a volume of **6 nL**, hydrodynamically injected into a separation capillary (40 µm i.d., 90 cm length) filled with 1% FA as a BGE. A coaxial sheath flow of 50% methanol with 0.1% (v/v) FA at a rate of 750 nL/min was used in CE-MS interface.



Hierarchical clustering of affected metabolites based on their participation in pathways containing differentially expressed genes. Pathways (rows) were clustered against metabolites (columns) monitored by CE−ESI-MS.
Yellow blocks indicate that a metabolite is involved in the corresponding pathway.
A total of 18 distinct metabolites were identified in the mouse hippocampus.

Knolhoff, A. M., Nautiyal, K. M., Nemes, P., Kalachikov, S., Morozova, I., Silver, R., Sweedler, J. V., *Anal Chem* 2013, *85*, 3136-3143.

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Patch clamp electrophysiology and CE−MS metabolomics for single cell characterization of brain cells (rat thalamus)

Ex vivo whole cell patch clamp as a sampling approach.
Intracellular material collected by applying additional negative pressure to puncture a passageway into the cell membrane and withdraw a small volume of **3 pL** of the **cytoplasm contents** into the patch clamp pipet for a small-volume assay using CE-MS.

nonburst firing TRN neuron

electrophysiological recording | photomicrograph LIF | extracted ion electropherogram

Combination of whole-cell patch clamp with single cell cytoplasm metabolomics.
Physiological activity of neurons and astrocytes correlated with their neurochemical state and quantitate changes in the cellular metabolome. **Striking cell to cell heterogeneity in the brain.**

microTOF or a maXis 4G Qq-ToF (Bruker Daltonics, Billerica)
positive ion mode
capillary length 65−70 cm, voltage 14−16 kV
sample injection volume of ~28 nL

Aerts, J. T., Louis, K. R., Crandall, S. R., Govindaiah, G., Cox, C. L., Sweedler, J. V., *Anal Chem* 2014, *86*, 3203-3208.

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Single Cell Analysis with Probe ESI-Mass Spectrometry: Detection of Metabolites at Cellular and Subcellular Levels

A. cepa cells

Assistant solvent spray

MS inlet

SPME probe

D.C.

Schematic of the probe ESI setup.
Microscopic image of the sampling procedure. Monolayer A. cepa cells placed on a glass slice. The dark cells alive, the light ones dead.
Coupling with MS: DC high voltage (+2.5 kV for the positive mode and −2.6 kV for the negative mode) applied to the probe. Assistant solvents directly sprayed onto the tip of the probe to generate electrospray.

Gong, X. Y., Zhao, Y. Y., Cai, S. Q., Fu, S. J., Yang, C. D., Zhang, S. C., Zhang, X. R., *Anal Chem* 2014, *86*, 3809-3816.

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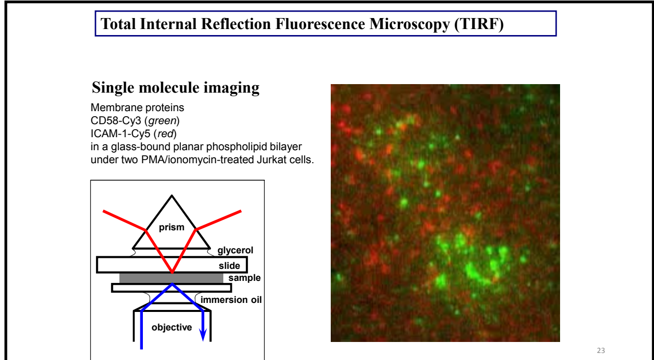
Optical methods

Total Internal Reflection Fluorescence Microscopy (TIRF)

Single molecule imaging

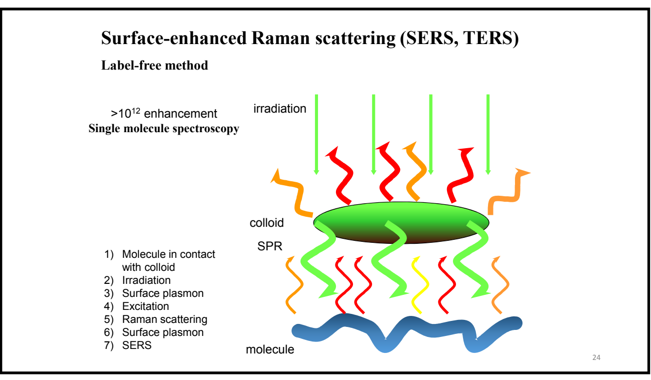
Membrane proteins
CD58-Cy3 (*green*)
ICAM-1-Cy5 (*red*)
in a glass-bound planar phospholipid bilayer under two PMA/ionomycin-treated Jurkat cells.



Surface-enhanced Raman scattering (SERS, TERS)

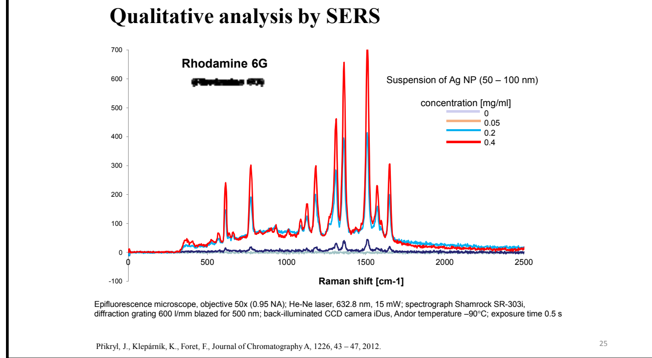
Label-free method

$>10^{12}$ enhancement
Single molecule spectroscopy



1) Molecule in contact with colloid
2) Irradiation
3) Surface plasmon
4) Excitation
5) Raman scattering
6) Surface plasmon
7) SERS

Qualitative analysis by SERS



Epifluorescence microscope, objective 50x (0.95 NA); He-Ne laser, 632.8 nm, 15 mW; spectrograph Shamrock SR-303i, diffraction grating 600 l/mm blazed for 500 nm; back-illuminated CCD camera iDus, Andor temperature –90°C; exposure time 0.5 s

Přikryl, J., Klepárník, K., Foret, F., Journal of Chromatography A, 1226, 43 – 47, 2012.

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Bioluminescence detection of caspase 3 - protease relevant in apoptosis

Caspase 3
- play a central role in the execution-phase of apoptosis
- increased amount of caspase indicates the point-of-no-return in apoptosis
- protease recognizing tetra-peptide sequence

Molecular mass ~ 33 000 Da

Asp-Glu-Val-Asp↓Gly

Interest of biologists, pharmacologists and physicians:

- ❖ Failure of apoptosis is one of the main contributions to tumor development, neurodegenerative and autoimmune diseases.
- ❖ Potential target for new drugs.
- ❖ New functions in cellular differentiation

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Bioluminescent determination of caspase 3 activity

Advantages:

- ❖ **one step mixing system**
- ❖ **no washing steps**
- ❖ **no immobilized agents**
- ❖ **convenient kinetics**
- ❖ **free aminoluciferin excluded**

Z-DEVD-luciferin
Z-DEVD
caspase 3
amino luciferin
luciferase Mg^{2+}
+ ATP + O_2
hν

D – aspartic acid
E – glutamic acid
V – valine

I. Chlastakova, M. Liskova, J. Kudelova, L. Dubska, K. Kleparnik, E. Matalova, Cellular and Developmental Biology, 48, 2012, 545-549.

Lišková M., Klepárník K., Matalová E., Hegrová J., Přikryl J., Švandová E., Foret F. Electrophoresis, 34, 2013, 1772-1777

E. Adamová, M. Lišková, E. Matalová, K. Klepárník Anal Bioanal Chem (2014) 406, 5389–5394

Chemiluminescence detection

- Limit of caspase 3 bioluminescent detection is ~ 0.2 of its content in apoptotic cell, i.e. < 1 fg.
- Contents of caspase 3 in individual apoptotic cells vary from ~ 50 to 250 thousand molecules.

CL chamber assembly with PMT

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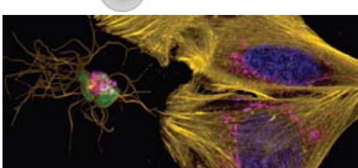
Quantum dot – donor in FRET sensor for confocal fluorescence caspase 3 microscopy

FRET - Foerster resonance energy transfer - „Angstrom optics“

- Huge absorption coefficient
- Broad excitation spectra
- Narrow emission spectra
- Low photobleaching

QD



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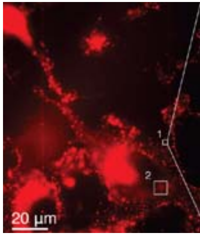
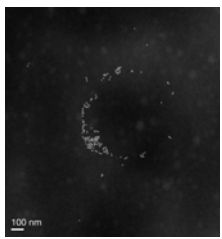
Correlative luminescence and electron microscopy of QD labeled proteins in cells





Transmembrane proteins: Fas receptor (CD95), molecular mass 48 kDa, and Fas ligand (CD178 - 40 kDa).
Molecular probe: conjugate of monoclonal antibody with QD
QD provides: dynamic image overview due to a bright luminescence (diffraction limit ~ 200 nm)
nanometer precise position due to a high electron density

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Single molecule detection

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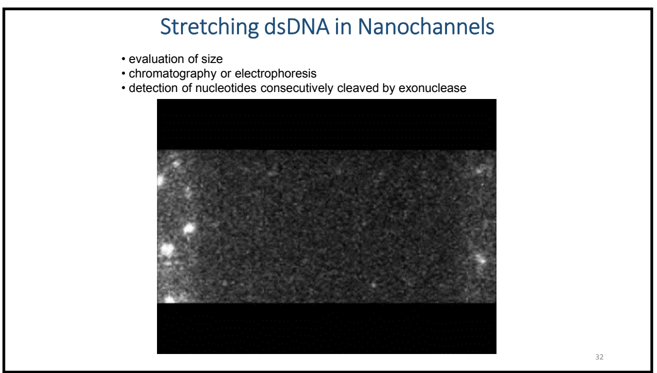

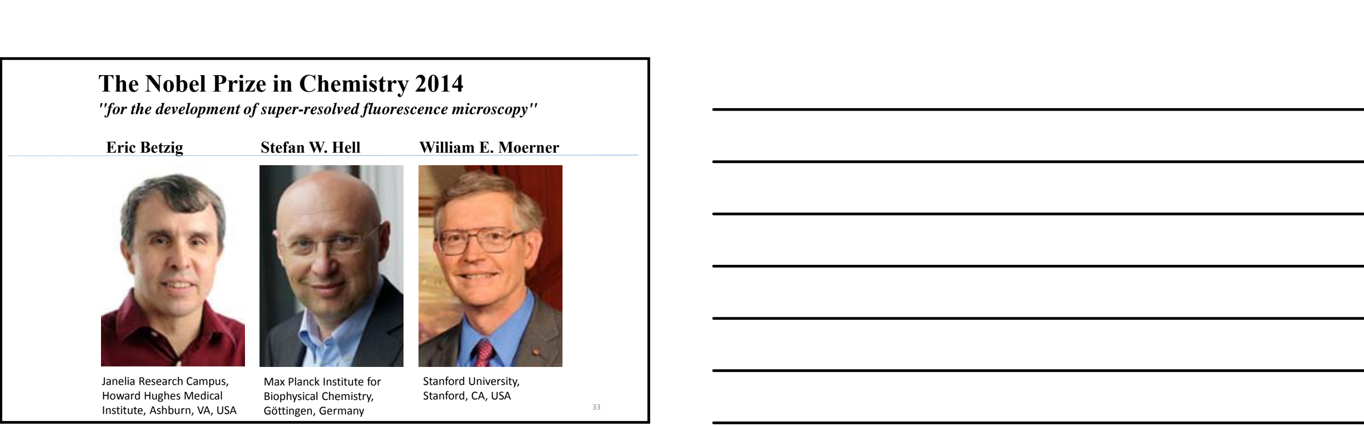

Super-resolution Optical Microscopy Nanoscopy
(resolution below diffraction limit)

STED - stimulated emission depletion
microscopy stimulated emission inactivate anuli around a point

STORM - stochastic optical reconstruction
random on and off states

PALM – photoactivated localization microscopy
photoswitchable fluorophores

SOFI - super-resolution optical fluctuation imaging
correlation function of fluctuations

A sufficiently low percentage of the probes are activated to allow the image of each fluorescing molecule to be seen separately

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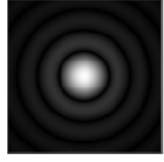
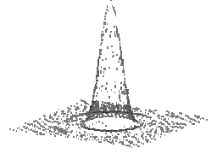
Abbe‘s Diffraction limit





$$d = \frac{\lambda}{2n \sin \alpha}$$

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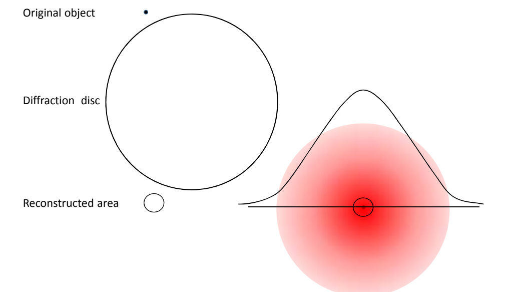
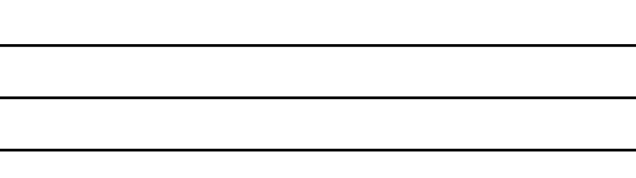
Point Spread Function





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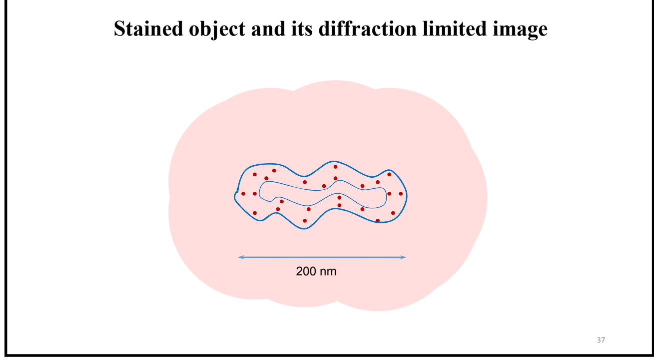
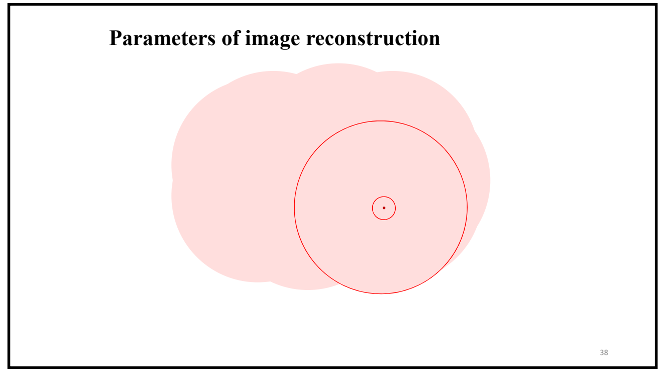
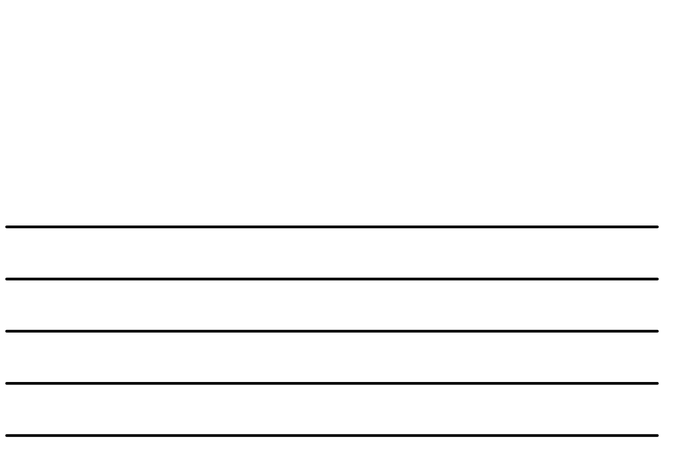
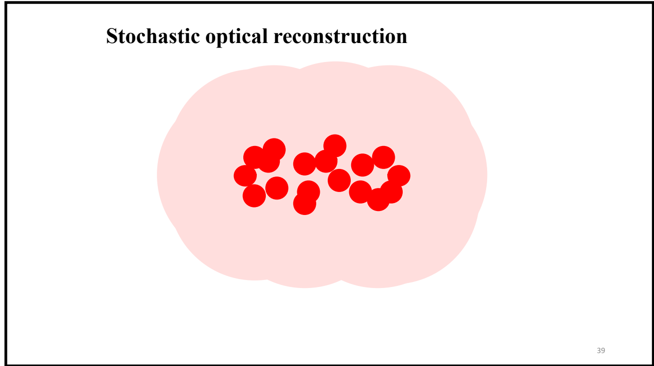
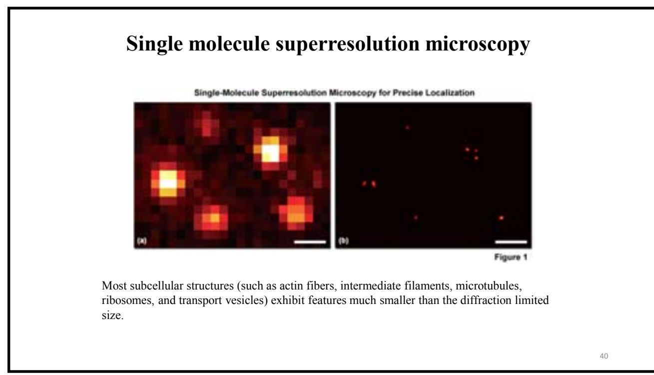
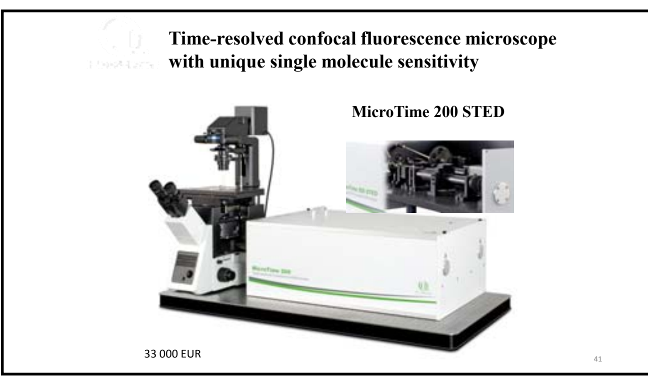

Stained object and its diffraction limited image
200 nm
37

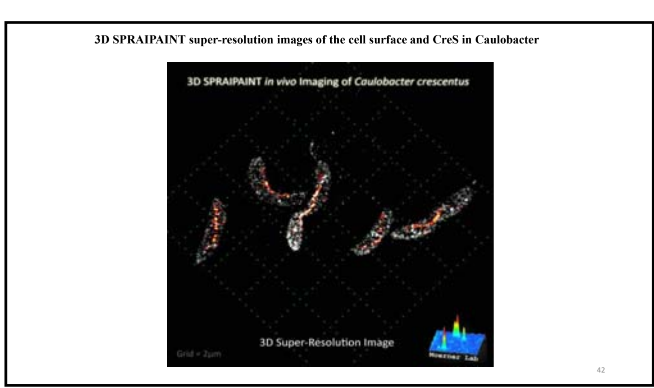

Parameters of image reconstruction
38



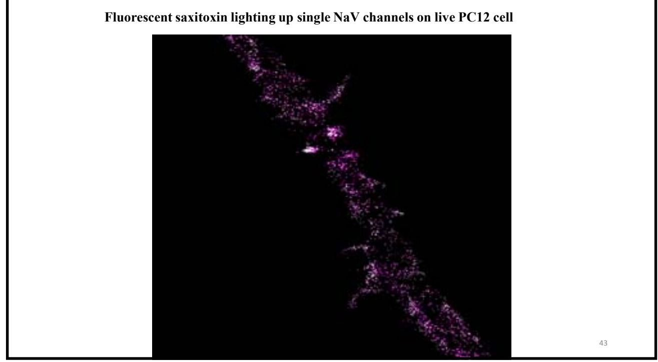

Stochastic optical reconstruction
39


Single molecule superresolution microscopy
Single-Molecule Superresolution Microscopy for Precise Localization
(a)
(b)
Figure 1
Most subcellular structures (such as actin fibers, intermediate filaments, microtubules, ribosomes, and transport vesicles) exhibit features much smaller than the diffraction limited size.
40


Time-resolved confocal fluorescence microscope with unique single molecule sensitivity
MicroTime 200 STED
33 000 EUR
41


3D SPRAIPAINT super-resolution images of the cell surface and CreS in Caulobacter
3D SPRAIPAINT in vivo Imaging of Caulobacter crescentus
3D Super-Resolution Image
42

Fluorescent saxitoxin lighting up single NaV channels on live PC12 cell



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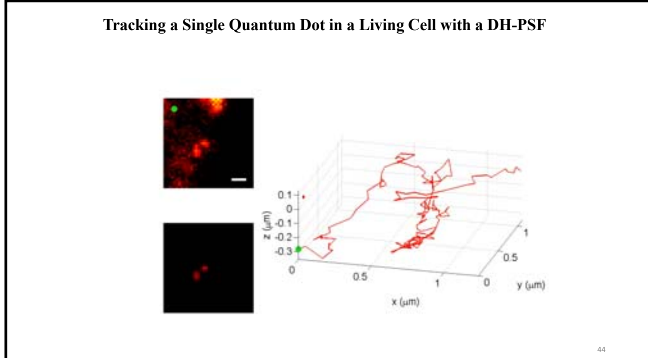
Tracking a Single Quantum Dot in a Living Cell with a DH-PSF



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Application of micro- and nanotechnologies in analytical chemistry provides:

- Methods with absolute sensitivity
- Space resolution - 25 nanometers
- Time resolution – ps (molecule – 6 GHz processor)
- Single molecule detection in individual cells (targeted proteomics)
- Label-free high-sensitivity methods
- High throughput multiplex parallel analyses

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Next generation DNA sequencing

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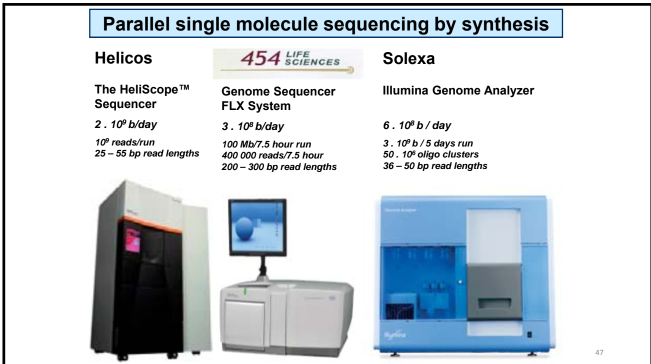
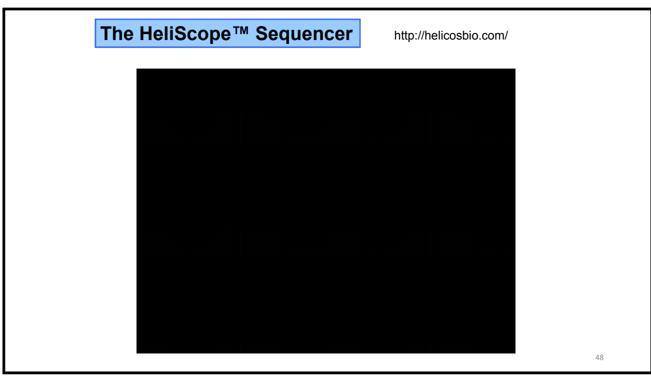
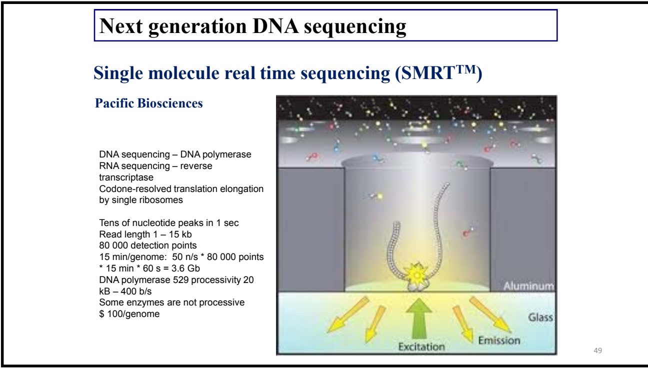


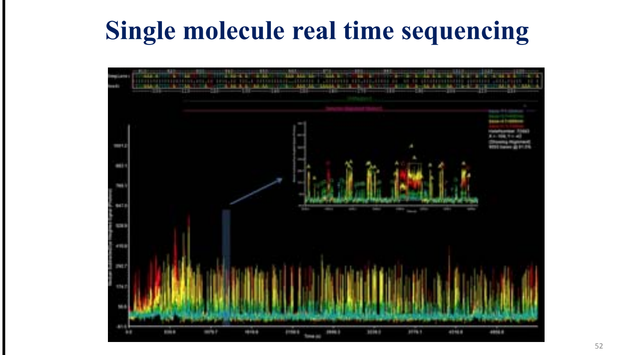



Next generation DNA sequencing
Single molecule real time sequencing (SMRT™)
Pacific Biosciences
DNA sequencing – DNA polymerase
RNA sequencing – reverse transcriptase
Codone-resolved translation elongation by single ribosomes
Tens of nucleotide peaks in 1 sec
Read length 1 – 15 kb
80 000 detection points
15 min/genome: 50 n/s * 80 000 points * 15 min * 60 s = 3.6 Gb
DNA polymerase 529 processivity 20 kB – 400 b/s
Some enzymes are not processive
$ 100/genome
Aluminum
Glass
Excitation
Emission
49

Pacific Biosciences
Single Molecule Real Time (SMRT™) DNA sequencing
50

PacBio RS instrument
51

Single molecule real time sequencing



Pacific Biosciences Read Length

New P5-C3 Sequencing Chemistry

Read Length Distribution

Number of Reads

Read Length

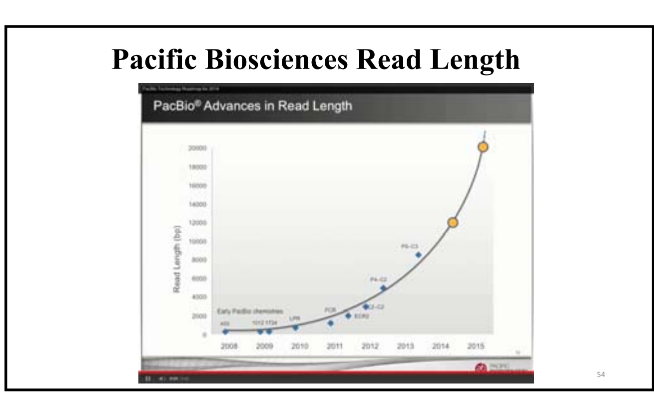
Typical Results

Read Length:

Average:	~ 8.5 kb
Maximum:	> 30 kb
Top 5% of reads:	> 18 kb
Half of data in reads:	> 10 kb
Data per SMRT® Cell:	~ 375 Mb

Based on data from a 20 kb size-selected E. coli library using a 180-minute movie.
Each SMRT Cell yields ~ 50,000 reads.

Pacific Biosciences Read Length



DNA sequencing development

2001: Genome draft of 5 individuals in 9 months
– more than billion $

2014: Complete human genome in an hour – ~100 $

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