Advances in dye development and microscopy for live cell super resolution microscopy with the Vutara 352

Rob J. Hobson, Ph.D., Applications Scientist

Swept-Field Confocal
Multiphoton
Super-Resolution
Luxendo Light-Sheet

Innovation with Integrity
Advances in dye development and microscopy for live cell super resolution microscopy with the Vutara 352

Fadi Jradi

Thien Vu
Outline

• Principles of Single Molecule Localization (SML)
• Vutara 352 Microscopy Hardware
• Example images
• Live single molecule super-resolution microscopy
• New approaches for single molecule live microscopy
• Quantitative Analysis in Localization Microscopy
The Diffraction Limit

Illustration: © Johan Jarnestad/The Royal Swedish Academy of Sciences
Principle of Single Molecule Localization
How do you get an isolated source in a densely labeled sample?

**Fluorescent Proteins**

1. Optically turn proteins from “off” state to “on” state
   - Photo-activatable

2. Optically switch emission spectrum, i.e. green to orange
   - Photo-convertible

**Organic Dyes**

1. Controlling fluorescent state of molecules through light-induced chemical reactions
   - (d)STORM

2. Transient binding of dye to cellular target
   - DNA-PAINT
Single Molecule Blinking
1. Fluorescent “on” state is optically controlled, only small subset “on” at a given instant in time

2. PSFs are sparse across a single camera frame, “diffraction-limit isolated”

3. Each PSF can be isolated with the data set

4. Perform localization algorithm on each PSF, such as non-linear least squares or maximum likelihood fitting

5. Extract spatial information on the location of the single molecule below the diffraction limit

6. Construct “super-resolution” image from analyzed data
Localization Microscopy Methodology
Localization Microscopy Reconstruction

Plane 1
Diffraction versus Super-Resolution

Diffraction & SML

SML

Cy3B TOM20, BSC1 cell line
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Vutara 352: Video Rate Super-Resolution

- 3D video-rate super-resolution microscope
- Precise 3D super-resolution (SML) : 20nm (X,Y) & 50nm (Z)
- Unique ability to image deep within samples
- Uniquely designed for SML imaging
- Easy to use, powerful analytical software suite
- Quantitative statistical analysis
Bruker Biplane Methodology

1 µm

X

Z

15

 Courtesy of Joerg Bewersdorf, Yale University
Single Molecule Blinking – Two Focal Planes
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SMLM - Biological Applications

Infectious Diseases

Cardiology

Reproduction

Developmental Biology

Cell Biology

Neuroscience
Developmental Biology - Thick Samples

Biological System: Whole mount Drosophila larva

Green: Alexa 488 Frizzled Receptor
Magenta: DyLight 649 Lamin C

Depth Animation
Sean Speese, OHSU

Orientation Animation
Multi-color Imaging in Cell Biology

Biological System: Cos 7 Cell
Green: Alexa 568 Complex IV
Red: Alexa 647 TOM 20
Image courtesy of Dr. Cliff Guy, St. Jude Children’s Research Hospital

Biological System: HeLa cell
Green: ATTO 488 tubulin
Red: Alexa 647 midbody protein
Blue: Cy3B midbody protein
Sundquist lab, University of Utah

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Fixed super resolution imaging

1. Cells are fixed
2. Membranes are permeabilized
3. Fluorescent antibodies label cell structures
4. Sample is imaged with high laser power and reducing agents

Advantage: High photon counts, stable imaging
Disadvantage: Dead
Live super resolution imaging

Movie of U2OS cells expressing tomm20::Halotag labeled with spontaneously blinking Janelia fluor 549 dye courtesy of Luke Lavis, Janelia, HHMI. 300 frames/frame, 20 ms exposure time.

1. Protein fluors, tags or vital dyes are used to visualize structures
2. Sample must be imaged with low laser power, and compatible buffer for long term imaging
3. Sample must be imaged with enough frequency to visualize your structure over time
Labeling strategies
Labeling strategies

Protein of interest (POI)

Protein fluor (e.g. mEOS)

How do we get the organic dyes on the protein of interest?

Protein of interest (POI)

Halotag/SNAPf tag

Halo/SNAP ligand + fluor

or

Halotag/SNAPf ligand + fluor
SMLM – Two types of live cell single molecule localization microscopy

Particle Tracking

Cellular Structures
3D Particle Tracking with the Vutara 352

Membrane Target

- Plane 1
- Plane 2

Model: Live A431 Cells
Label: QDot655-EGFR
Speed: 50 frames/s

Intracellular Target

Model: Live BSC1 cells
Label: Cy3B labeled mRNA
Speed: 200 frames/s
Live single molecule localization microscopy: How does it work in practice?
Live single molecule localization microscopy: How does it work in practice?

Cellular structures are dynamic
Live single molecule localization microscopy: How does it work in practice?

- To visualize cellular dynamics by SMLM we then need to bin a number of frames together to make a single image ‘Frame’
- We therefore need to sample at a high enough frequency to be able to rebuild the structure we are imaging
Live single molecule localization microscopy: How does it work in practice?

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High Speed Imaging via sCMOS

200 fps, 60,000 frames

Z-stack depth color coded

200 fps, 48,000 frames

Alexa Fluor 647 labeled TOM-20 (Red) and Cy3B labeled tyrosinated tubulin (Green) in fixed BSC1 cells.

Depth-color coded image of Alexa Fluor 647 labeled TOM-20 in BSC1 cells.

Images courtesy of H. Arami & M. Kanow (U. Washington) and S. Cameron (UC Davis)
High Speed Imaging with sCMOS Camera

800 fps
2000 frames
2.5 s

2500 fps
10000 frames
4 s

Alexa Fluor-647 labeled alpha-tubulin (Red)
Live single molecule localization microscopy: How does it work in practice? – Too little label…

- You must have enough localizations/frame to label your structure of interest.
- To prevent artifacts like this you need to either increase the frame count (thus slowing the overall frame rate of the movie) or increase the sampling frequency (which might reduce the photon count)
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Live single molecule localization microscopy: How does it work in practice? Slow sampling.
Live super resolution imaging

1. Cells are alive
2. Membranes are intact
3. Protein fluoros, tags or vital dyes are used to visualize structures
4. Sample must be imaged with low laser power, and compatible buffer for long term imaging

Movie of U2OS cells expressing tomm20::Halotag labeled with spontaneously blinking Janelia fluor 549 dye courtesy of Luke Lavis, Janelia, HHMI. 300 frames/frame, 20 ms exposure time.
Live vs fixed imaging

**Fluorescent Proteins**

Pros:
- Genetically encoded
- Standard sample prep
- Live cell compatible

Cons:
- Low photon budget (100’s)
- Homogenous spectra

**Organic Dyes**

Pros:
- High photon budget (1000’s)
- Heterogeneous spectra

Cons:
- Not always cell permeable
- Require high laser powers and toxic buffers

Cartoon of structure of GFP - Protein Data Bank
Chemical Structure of Rhodamine
Fluorescent Proteins

1. Optically turn proteins from “off” state to “on” state
   - Photo-activatable – e.g. PA-GFP, PA-mCherry etc
2. Optically switch emission spectrum, i.e. green to orange
   - Photo-convertible eg. Dendra, kaede, mEOS etc…

Organic Dyes

1. Controlling fluorescent state of molecules through light-induced chemical reactions
   - (d)STORM: AF647, CF568 (Biotium)
2. Caged dyes
   - PA-JF549, PA-JF646, etc…
3. Blinking dyes
   - HM-SiR, SB-JF549
SMLM – Live Cell Biological Applications

Protein Fluors

Organic Dyes
Live-cell Imaging using mEos3.2

- Biological System: Live HeLa Cell
- Label: mEos3.2-clathrin light chain
- Imaged at 600 fps for 58 s
- 2 seconds per SR image
- Imaged in PBS

Adapted from Huang et al. Nat. Meth. 10, 653-658 (2013)
Live-cell Fast Imaging using Organic Dyes

- Biological System: LiveEA.Hy926 Cell
- Label: AlexaFluor 647 labeled transferrin
- Imaged at 1600 fps
- Super-resolution images were reconstructed from sequential sets of 50 frames (31-ms acquisition time or 32 super-resolution images per second)
- Cells were imaged DMEM (high glucose, phenol red–free) supplemented with 2-beta mercaptoethanol, glucose oxidase and catalase at room temperature.
- Scale bars: 500 nm

Adapted from Huang et al. Nat. Meth. 10, 653-658 (2013)
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SMLM – Live Cell Biological Applications

New approaches for live cell dye development

Multi color imaging
Live vs fixed imaging

Fluorescent Proteins

Pros:
- Genetically encoded
- Standard sample prep
- Live cell compatible

Cons:
- Low photon budget (100’s)
- Homogenous spectra

Organic Dyes

Pros:
- High photon budget (1000’s)
- Heterogeneous spectra

Cons:
- Not always cell permeable
- Require high laser powers and toxic buffers
Live vs fixed imaging

Fluorescent Proteins

Organic Dyes

**Ideal dye:**
- Spontaneously blink under relatively low laser power
- Require little or no external buffer requirements
- Cell Permeable
- Photostable
- Lots of photons
Tetramethylrhodamine (TMR)

Credit to Thien Vu for slide

Luke Lavis
Azetidine rings improve quantum efficiency and quantum yield

Tetramethylrhodamine (TMR)  \( \Phi = 0.41 \)

JF549  \( \Phi = 0.88 \)

...but they don’t blink very well in mammalian cells...

Credit to Thien Vu for slide

Luke Lavis
Photoactivatable derivatives of the JF dyes improve single molecule localization

Organic Dyes – PA-JF646

16: R = HaloTag ligand
17: R = SNAP-tag ligand

Probes stay on for ~20-60 ms
Median photon count of ~700 photons
14 nm median radial precision
39 nm median axial precision

6 kW/cm²
With 405nm activation

Orthogonal dye available:
PA–JF-549

Dyes available for purchase from Tocris

Movie of U2OS cells expressing tomm20::Halotag labeled with PA-JF646 dye courtesy of Luke Lavis, Janelia, HHMI.
Photoactivatable derivatives of the JF dyes improve single molecule localization

**Organic Dyes – PA-JF646**

![Chemical structure of PA-JF646](image)

**16**: R = HaloTag ligand  
**17**: R = SNAP-tag ligand

By Grimm et al. 2016 Nature Methods

**Orthogonal dye available:**  
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Movie of U2OS cells expressing tomm20::Halotag labeled with PA-JF646 dye courtesy of Luke Lavis, Janelia, HHMI.  
150 frames/frame, 20 ms exposure time. Colored by depth

Probes stay on for ~20-60 ms  
Median photon count of ~700 photons  
14 nm median radial precision  
39 nm median axial precision  

6 kW/cm²  
With 405nm activation
PA-JF dyes still require relatively high laser power and UV to activate them resulting in toxicity after long term imaging.

**Movie of U2OS cells expressing tomm20::Halotag labeled with PA-JF646 dye courtesy of Luke Lavis, Janelia, HHMI.**

300 frames/frame, 20 ms exposure time. Colored by depth.

With 405nm activation

6 kW/cm²
Live vs fixed imaging

Fluorescent Proteins

Organic Dyes

Ideal dye:
- Spontaneously blink under relatively low laser power
- Require little or no external buffer requirements
- Cell Permeable
- Photostable
- Bright
How to get dyes to spontaneously blink?

Luke Lavis & Fadi Jradi
Spontaneously blinking JF dyes

Spontaneously blinking Janelia Fluor 549 (SB-JF549), from Luke Lavis, HHMI, Janelia

Credit to Thien Vu for slide

Luke Lavis & Fadi Jradi
Under physiological conditions spontaneously blinking Janelia fluor 549 (SB-JF549) is mostly closed.

SB-JF549, from Luke Lavis, HHMI, Janelia

Credit to Thien Vu for slide
Live Spontaneously Blinking SB-JF549

Spontaneously blinking Janelia Fluor 549, from Luke Lavis, HHMI, Janelia

Mean photon ~ 600
On-time: 10-20 ms

20 um x 20 um FOV
0.75 kW/cm²

Credit to Thien Vu for slide
Spontaneously blinking SB-JF549

SB-JF549, from Luke Lavis, HHMI, Janelia

**Ideal dye:**
- Spontaneously blink under relatively low laser power
- Require little or no external buffer requirements
- Cell Permeable
- Photostable
- Can be conjugated to SNAP or Halo ligands so they can be used to label proteins in cells.
Live Spontaneously Blinking SB-JF549

Spontaneously blinking Janelia Fluor 549, from Luke Lavis, HHMI, Janelia

100 binned frames/frame (20 msec exposure). ~4.5 million localizations.
U2OS cells expressing tomm20::Halo::SB-JF549
Imaged constantly for 30 minutes

0.75 kW/cm²

9 μm
Live 3D stacks using SB-JF549

2000 frames/Z stack (5 µm Z stack) U2OS cells expressing tomm20::Halo::SB-JF549
5 µm Z stack, 0.75 kW/cm²

SB-JF549, from Luke Lavis, HHMI, Janelia
This approach also works with other types of dyes.

SB-Alexa Fluor 594, from Luke Lavis, HHMI, Janelia

Particle Frame Histogram

Particle Parameter Histogram

Color 2 (P.)

SB-Alexa Fluor 594

Luke Lavis & Fadi Jradi
Spontaneously blinking dyes require low laser intensities for single molecule localization microscopy

Standard organic dyes

![Chemical structure of standard organic dye]

Typical laser powers required for blinking: 15 - 6 kW/cm²

Spontaneously blinking dyes

![Chemical structure of spontaneously blinking dye]

Typical laser powers required for blinking: 0.7 kW/cm²

Take home: you need an order of magnitude less power to do single molecule localization microscopy with spontaneously blinking dyes
Two color live cell super resolution microscopy

Fluorescent Proteins

- PA-GFP
- mEOS variants
- Skylan-S
- etc...

Organic Dyes

- Enzyme tags: Halo (Promega), SNAPf or CLIPf (NEB), etc

- Dyes: PA-JF646 and PA-JF549 (Tocris), HMSiR (Goryo), SB-JF549 (Lavis), etc
CAAX- photoactivatable GFP

Synaptobrevin-HALO: SB-549

Data courtesy of Thien Vu and Prof. Erik Jorgensen, HHMI, Univ. of Utah

SB-JF549, from Luke Lavis, HHMI, Janelia
CAAX- photoactivatable GFP

Synaptobrevin-HALO: SB-549

Data courtesy of Thien Vu and Prof. Erik Jorgensen, HHMI, Univ. of Utah

SB-JF549, from Luke Lavis, HHMI, Janelia
CAAX- photoactivatable GFP

Synaptobrevin-HALO: SB-549

Credit to Thien Vu for slide SB-JE549, from Luke Lavis, HHMI, Janelia
CAAX- photoactivatable GFP
Synaptobrevin-HALO: SB-549

Credit to Thien Vu for slide
SB-JF549, from Luke Lavis, HHMI, Janelia

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Optical Super-Resolution in the 21st Century

- Numerous advancements in super-resolution techniques
  - Near-field scanning optical microscopy (NSOM)
  - 4 Pi Microscopy
  - Structured Illumination
  - Stimulated Emission Depletion Microscopy (STED)
  - RESOLFT
  - Localization Microscopy Methods
    - (F)PALM
    - STORM
    - dSTORM
    - Ground State Depletion

Localization methods allow for experimental quantification directly from the molecular positions in the sample.
What Kind of Measurements are Useful for SML?

- SML is localizing individual molecules — provides the opportunity to make measurements based upon localization positions, calculate densities, and study the relationships of units of different species within biological structures.
- Types of measurements available within the SRX software:
  - **Spatial distribution**: Provides a variety of tools for analyzing spatial distribution relationships of particles including Ripley’s K, Pair correlation and Nearest Neighbors.
  - **Cluster Analysis**: Counts clusters, cluster sizes, cluster densities, intra/inter-cluster relationships.
  - **Co-localization**: Provides statistical measures on relationships between particles or clusters of two different labels.
  - **Resolution Analysis**: Quantifies resolution for images derived from localized data sets.
  - **Live Cell Analysis**: Tracking of fluorophores in live cell experiments with mean squared displacement and angular displacement analysis.
  - **Control Data**: Generate statistically random data to use as comparative data controls.
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Live Cell Imaging of BSC1 cells labeled with AF647 transferrin — single trajectory maps
Particle Tracking Algorithms

Localization Data

Particle Tracking

Max travel per frame = 300 nm
Live Cell Analysis

Time-lapse movie of Alexa Fluor 647 labeled transferrin receptor moving through cell.

Live cell analysis can track particle displacement, showing movement over time, and generate distance, velocity and angular distribution histogram plots.
Photoactivatable stay on for multiple frames and can be used for single particle tracking analysis.

Organic Dyes – PA-JF646

16: R = HaloTag ligand
17: R = SNAP-tag ligand

Particle tracking in living cells

Organic Dyes – PA-JF646

\[
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Movie of U2OS cells expressing tomm20::Halotag labeled with PA-JF646 dye courtesy of Luke Lavis, Janelia, HHMI.
150 frames/frame, 20 ms exposure time. Colored by depth

Grimm et al. 2016 Nature Methods
Photoactivatable derivatives of the JF dyes allow live cell single particle tracking

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Vutara SML Quantitative Analysis

- Live and fixed cell imaging
  - Fastest imaging, unique ability to image in 3D far from the coverslip
- Quantitative data in a matter of minutes
  - SRX localizes data in real-time, allows visualization and analysis in real-time as data is acquired
- SML modality allows scientific investigations at molecular level
  - Unique among far-field optical super-resolution methodologies
- Vutara SRX software allows user to image, visualize and quantify in minutes - improve workflow, experimental testing and throughput

Acquire & Localize → Visualize → Analyze
Questions?

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