Live Cell Super-Resolution Imaging

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Atomic Force Microscopy
3D Optical Microscopy
Fluorescence Microscopy
Tribology
Stylus Profilometry
Nanoindentation
Outline

- Super-Resolution Microscopy
- Principle of Single Molecule Localization (SML)
- Factors crucial for successful live cell SML imaging
- Vutara 350- Live Cell Imaging Ready Technology
- Sample Prep Optimization
- New Dye Development
Evolution of Super-resolution Microscopy

<table>
<thead>
<tr>
<th></th>
<th>Confocal</th>
<th>SIM</th>
<th>STED</th>
<th>Single-molecule localization (SML)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XY resolution</td>
<td>250 nm</td>
<td>100-130 nm</td>
<td>40-60 nm</td>
<td>20-30 nm</td>
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<tr>
<td>Z resolution</td>
<td>500-700 nm</td>
<td>250-350 nm</td>
<td>100-700 nm</td>
<td>50-80 nm</td>
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</table>
Principle of Single Molecule Localization (SML)

Images are captured from two focal planes simultaneously on the Vutara microscope (f)PALM, STORM, dSTORM, GSDIM
Factors Essential for Successful Live Cell Super-resolution Imaging

- Cutting Edge Technology-Hardware and Software Features
- Speed of imaging-To capture dynamics at the right temporal scale
- High power lasers-control photoswitching kinetics
- Real time localization of molecules-for quick results to determine course of experiment
- Sample Prep Optimization-to get the best spatial and temporal resolution
- Live cell compatible fluorophores-to have choice of cell permeable fluorophores and ones that switch in the absence of thiols
The Technology: Vutara 350 Video Rate Super-Resolution Microscope

- Fastest 3D super-resolution microscope on the market
- Only 3D video-rate super-resolution microscope
- High speed imaging with sCMOS camera-up to 3000 fps
- High power laser to drive fast switching kinetics
- Precise 3D super-resolution (SML) : 20nm (x,y) & 50nm (z)
- Easy to use, yet powerful software with real time localization
- Multi-emitter fitting
## Comparison between sCMOS and EMCCD Cameras

<table>
<thead>
<tr>
<th></th>
<th>sCMOS (ORCA-Flash4.0)</th>
<th>EMCCD (iXon Ultra 897)</th>
</tr>
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<tbody>
<tr>
<td><strong>Field of View</strong></td>
<td>2048 x 2048 pixels</td>
<td>512 x 512 pixels</td>
</tr>
<tr>
<td><strong>Pixel size</strong></td>
<td>6.5 x 6.5 µm²</td>
<td>16 x 16 µm²</td>
</tr>
<tr>
<td><strong>Frame rate</strong></td>
<td>100 fps (full frame)</td>
<td>56 fps (512 x 512)</td>
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<tr>
<td></td>
<td><strong>400 fps</strong> (512 x 512)</td>
<td></td>
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<tr>
<td><strong>QE @ 600 nm</strong></td>
<td>72%</td>
<td>48% (effective)</td>
</tr>
<tr>
<td><strong>Readout Noise</strong></td>
<td>Pixel dependent</td>
<td>Negligible</td>
</tr>
</tbody>
</table>
ResEnhanced Technology for sCMOS

Adapted from Huang et al. Nat. Meth. 10, 653-658 (2013)
Comparison of Achievable Localization Precision

- Signal to noise ratio (SNR) of sCMOS is favorable to EMCCD at >6 photons/pixel
- Typical SML conditions *ca.* $10^2$ photons/pixel or higher

Adapted from Huang et al. Nat. Meth. 10, 653-658 (2013)
High Speed Imaging with sCMOS Camera

800 fps
2000 frames
2.5 s

800 fps
2000 frames
2.5 s

2500 fps
10000 frames
4 s

Alexafluor-647 labeled alpha-tubulin (Red) and Cy3B labeled TOM20 (Green) in BSC1 cells. Examples shown in fixed cells but can be used in live cell imaging.
High Power Lasers

Jones et al. Nat. Meth. 8, 499-505 (2011)

488 – 1000mw
561 – 1000mw
640 – 1000mw
750 – 1000mw
SML at High Speed: Multi-emitter Fitting

Adapted from Huang et al. Nat. Meth. 10, 653-658 (2013)

Single emitter fitting-Separate PSFs

Multi-emitter fitting-Overlapping PSFs
Software: Parallel Processing

• Highly Parallel Architecture
• GPU + Multi-threaded CPU
• Scalable architecture – up to 40TB
• Enable real time localization while acquiring data
• Multi-emitter fitting
Sample Labeling Choices for SML Imaging

- Organic dyes or Genetically encoded fluorescent proteins
- Organic dyes generally preferred for SML labeling over fluorescent proteins since they emit more photons.
- Fluorescent proteins are live cell compatible
# Single Molecule Localization Probes

## Preferred Organic Dyes

<table>
<thead>
<tr>
<th>Excitation Laser Line (nm)</th>
<th>Dye</th>
<th>Excitation Maximum (nm)</th>
<th>Emission Maximum (nm)</th>
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<tbody>
<tr>
<td>488</td>
<td>ATTO 488</td>
<td>501</td>
<td>523</td>
</tr>
<tr>
<td></td>
<td>Alexa 488</td>
<td>495</td>
<td>519</td>
</tr>
<tr>
<td>561</td>
<td>Cy3B</td>
<td>559</td>
<td>570</td>
</tr>
<tr>
<td></td>
<td>Alexa 568</td>
<td>578</td>
<td>603</td>
</tr>
<tr>
<td></td>
<td>Alexa 555</td>
<td>555</td>
<td>580</td>
</tr>
<tr>
<td>640</td>
<td>Alexa 647</td>
<td>650</td>
<td>665</td>
</tr>
<tr>
<td></td>
<td>Cy5</td>
<td>649</td>
<td>670</td>
</tr>
<tr>
<td></td>
<td>DyLight 650</td>
<td>652</td>
<td>672</td>
</tr>
<tr>
<td>750</td>
<td>Alexa 750</td>
<td>749</td>
<td>775</td>
</tr>
<tr>
<td></td>
<td>DyLight 755</td>
<td>754</td>
<td>776</td>
</tr>
<tr>
<td>Probe</td>
<td>Type</td>
<td>(\lambda_{PA}) (nm)</td>
<td>(\lambda_X) (nm)</td>
</tr>
<tr>
<td>----------------</td>
<td>------------</td>
<td>-------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>PSCFP2</td>
<td>0→A (Irrev)</td>
<td>Violet (~400)</td>
<td>490</td>
</tr>
<tr>
<td>PA-GFP</td>
<td>0→A (Irrev)</td>
<td>Violet</td>
<td>504</td>
</tr>
<tr>
<td>Dronpa</td>
<td>0→A (Rev*)</td>
<td>*activ. w violet quench w blue</td>
<td>503</td>
</tr>
<tr>
<td>Dendra2</td>
<td>A→B (Irrev)</td>
<td>Violet-Blue</td>
<td>553</td>
</tr>
<tr>
<td>EosFP</td>
<td>A→B (Irrev)</td>
<td>Violet</td>
<td>569</td>
</tr>
<tr>
<td>Kaede</td>
<td>A→B (Irrev)</td>
<td>Violet</td>
<td>572</td>
</tr>
<tr>
<td>KikGR</td>
<td>A→B (Irrev)</td>
<td>Violet</td>
<td>583</td>
</tr>
<tr>
<td>PAmCherry</td>
<td>0→A (Irrev)</td>
<td>Violet</td>
<td>564</td>
</tr>
</tbody>
</table>
Fast 2-Color Live Super-Resolution Imaging Reveals Dynamic Associations

HA Moves Along Actin-Rich Membrane Regions in Fibroblasts at 37°C

Gudheti et al. Manuscript in preparation
3-Color FPALM of Dendra2-HA, PAmCherry-Actin, and PAmKate-Transferrin Receptor

Dendra2-HA (2,788 mol)
PAmCherry-Actin (7,316 mol)
PAmKate-TfR (2,967 mol)

Gunewardene et al. Super-resolution imaging of multiple fluorescent proteins with highly overlapping emission spectra in living cells (2011) Biophysical J 101
Three color super-resolution imaging of multiple fluorescent proteins in living cells

Gunewardene et al. Super-resolution imaging of multiple fluorescent proteins with highly overlapping emission spectra in living cells (2011) Biophysical J 101
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.

Adapted from Huang et al. Nat. Meth. 10, 653-658 (2013)
Live Cell Imaging of BSC1 cells Labeled with AF647 Transferrin (3000 frames total imaged)
Live-Cell Imaging of Direct-Labeled Cellular DNA

- 1 mM ascorbic acid, 10% glucose, glucose oxidase and catalase in Leibowitz medium, pH 7.2
- 8000 frames
- 30 ms acquisition time

Super-resolution fluorescence imaging of organelles in live cells with photoswitchable membrane probes

(A) the plasma membrane labeled with Dil in a hippocampal neuron (15 sec)  
(B) mitochondria labeled with MitoTracker Red in a BS-C-1 cell (10 sec)  
(C) the ER labeled with ER-Tracker Red in a BS-C-1 cell (10 sec)  
(D) lysosomes labeled with LysoTracker Red in a BS-C-1 cell (1 sec)  

Scale bars, 1 μm.

Shim et al. PNAS. 109, 13978-13983 (2012)
Super-resolution imaging in live Caulobacter crescentus cells using photoswitchable EYFP

Figure 2 | PALM images of EYFP-MreB in C. crescentus cells. (a,b) Banded structure in a stalked cell. (c,d) Midplane ring in a predivisional cell. Fluorescence PALM images are shown in a and c. The PALM images in b and d are the same cells as in a and c, respectively, overlaid on a reversed-contrast white-light transmission image of the cell. Scale bars, 300 nm.

Imaging Without Switching Buffer

- Biological System: Vero Cell
- **Green**: Cage fluorescent dye 505 clathrin
- **Red**: Rhodamine spiroamide 565 Tubulin
- Imaged in PBS
Combining the best of organic dyes and Fluorescent Proteins: SNAP, CLIP and Halo Tags

- New labeling technologies are being developed to exploit the best features of organic dyes and genetically encoded proteins

Combining the best of organic dyes and Fluorescent Proteins: SNAP, CLIP and Halo Tags

Imaging proteins inside cells with fluorescent tags


Variety of SNAP, CLIP and Halo dyes are commercially available

<table>
<thead>
<tr>
<th>SNAP tag Dyes</th>
<th>CLIP tag Dyes</th>
<th>Halo tag Dyes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEB</td>
<td>NEB</td>
<td>Promega</td>
</tr>
<tr>
<td>SNAP-Cell® 505-Star</td>
<td>CLIP-Cell™ 505 Star</td>
<td>HaloTag® TMR</td>
</tr>
<tr>
<td>SNAP-Cell® TMR-Star</td>
<td>CLIP-Cell™ TMR-Star</td>
<td>HaloTag® Alexa Fluor® 488</td>
</tr>
<tr>
<td>SNAP-Cell® 647-SiR</td>
<td>CLIP-Surface™ 488</td>
<td>HaloTag® Alexa Fluor® 660</td>
</tr>
<tr>
<td>SNAP-Surface® 488</td>
<td>CLIP-Surface™ 547</td>
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</tr>
<tr>
<td>SNAP-Surface® 549</td>
<td>CLIP-Surface™ 647</td>
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<td>SNAP-Surface® 594</td>
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<td>SNAP-Surface® 649</td>
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<td>SNAP-Surface® Alexa Fluor® 546</td>
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<tr>
<td>SNAP-Surface® Alexa Fluor® 647</td>
<td></td>
<td></td>
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<tr>
<td>SNAP-Surface® Alexa Fluor® 488</td>
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</tr>
</tbody>
</table>

Fast, three-dimensional super-resolution imaging of live cells

Figure 3 | Two-color 3D STORM images of CCPs and transferrin in live cells. (a) Conventional image of CCPs and transferrin in a live cell. CCPs were labeled with Alexa647 via a SNAP tag (magenta) and transferrin was directly labeled with Alexa568 (green). (b) A 3D STORM image x-y projection of the same area taken in 30 s. (c,d) STORM images of CCPs indicated in b: x-y cross-section near the plasma membrane (left), x-z cross-section cutting through the middle of the invaginating CCP (middle), and corresponding x-z cross-section of the clathrin channel only (right). Scale bars, 500 nm (a,b) and 100 nm (c,d).

Jones et al. Nat. Methods. 8, 499-505 (2011)
Live-cell dSTORM with SNAP-tag fusion proteins

(a) Fluorescence image of histone H2B proteins in a COS-7 cell stained with SNAP-Cell TMR-Star (1 μM). Scale bar, 5 μm.
(b) Fluorescence image of the same cell but with 532-nm excitation of ~1 kW cm−2, which induced photoswitching
(c) dSTORM image reconstructed from 10,000 images (acquired at 50 Hz without additional irradiation at 405 nm).

Klein et al. Nat. Methods. 8, 7-9 (2011)
Development of New Dyes-Crucial for Driving Live Cell Super-Resolution Imaging Forward

A spontaneously blinking fluorophore based on intramolecular spirocyclization for live-cell super-resolution imaging
Various dyes: Rhodamine derivatives
Development of New Dyes—Crucial for Driving Live Cell Super-Resolution Imaging Forward

A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins
Dye: SiR (Silicon Rhodamine)
Imaging the ventral nerve cord of *C. elegans* in an intact animal - Fixed after live labeling

The ventral nerve cord is a complex mixture of cholinergic and GABAergic synapses. It would be impossible to see individual synapses without super-resolution microscopy.

Image courtesy of Claire Liu, Dr. Rob Hobson, Erik Jorgensen Lab, Univ. of Utah
Imaging cholinergic neuromuscular synapses in intact animals—Fixed after live labeling

**Green** = synaptotagmin-1::SNAPf::TMRstar (synaptic vesicles)

**Red** = RIM::Halotag::SiR (Active zone)

Image courtesy of Dr. Rob Hobson, Erik Jorgensen Lab, Univ. of Utah
Summary

- Vutara 350 super-resolution microscope is live cell imaging ready - Hardware and software have been optimized for this purpose
- Sample prep optimization is crucial for achieving good live cell super-resolution imaging
- Choose the right imaging conditions (imaging buffer compatible with dyes, laser powers, acquisition time, and number of frames)
- Development of new dyes and labeling techniques is what is going to propel the field forward and drive new scientific discoveries