Super-resolution and single molecule imaging of bacteria

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These techniques dramatically increase resolution and allow us to probe the behaviour of single proteins in live cells.

Revolutionary throughout biology.

But particularly useful in bacteria due to their small size and their relative simplicity.
Diffraction poses serious problems in bacteria...

Diffraction limits the resolution of light microscopy: In practice this is a serious limitation!

Huang et al, Cell (2010)
Super-resolution microscopy resolves this problem

Diffraction limits the resolution of light microscopy:

In practice this is a serious limitation!

Super-resolution microscopy to the rescue...

Single molecule microbiology

Biology works at the single molecule level!

Examples:
• Chromosome is a single molecule!
• Gene expression is performed by a single molecule nanomachine - RNA polymerase
• Cell wall remodelling is performed by single multi-enzyme complexes

Nguyen et al PNAS 2015
**Single molecule microbiology**

Different copies of a protein will be in multiple different states in the cell. Eg, RNAP bound/ unbound to DNA:

“Ensemble” methods average over these different states. To get accurate information we need to measure one molecule at a time.
Super-resolution methods

A. PALM/STORM
Key concept: photoactivation / photoswitching
- Key concept: light
- Key concept: bright state
- Key concept: dark state
- Key concept: PSF: 200-300 nm

B. SIM
Key concept: moiré effect
- Key concept: fine structure
- Key concept: applied pattern
- Key concept: moiré fringes

C. STED
Key concept: stimulated depletion
- Key concept: excitation spot
- Key concept: depletion spot
- Key concept: detection spot

Resolution:
- PALM/STORM: 20 nm
- SIM: 100 nm
- STED: ~50 nm

Coltharp & Xiao, Cell Microbiol 2012
Localization microscopy: principle

**A** PALM/STORM

**Key concept:** photoactivation / photoswitching

- **Dark state**
- **Bright state**

- **Light**

**PSF:** 200-300nm

**Acquisition sequence:**

1,000-10,000 frames

**Localize & superimpose**
It’s all about making fluorophores blink!

Photoactivatable fluorescent proteins:

Photoswitchable organic dyes
... and then finding their centres

Stochastic Optical Reconstruction Microscopy (STORM)/Photoactivation Localization Microscopy (PALM)

STORM over the Eiffel Tower

Henriques et al., Nat. Methods (2010)
Spatial resolution

XY: 25 nm
Z: 100 nm
Resolution

Spatial resolution
XY: 25 nm
Z: 100 nm

Time (typ.): 3 – 5 mins
Time (best): 2 s (FPs), 30 ms (dyes)

Advantages:
- Highest resolution of SR microscopies
- Single molecule information

Disadvantages:
- Requires high laser powers
  → phototoxicity problems
Localization microscopy: applications

RNA polymerase
FtsZ
CheY
DNA polymerase
Crescentin

Endesfelder, Finan, Holden et al., Biophys J. (2013)
Holden et al, PNAS (2014)
Greenfield et al, Plos Biol (2009)
Lew et al, PNAS (2011)
Uphoff et al, PNAS (2013)
FtsZ ultrastructure

Diffraction limited imaging of the cell division cytoskeletal “Z-ring” look continuous:

Consistent with the idea of a force generating constrictive ring:
Super-resolution suggests a patchy Z-ring

*C. crescentus* 3D PALM

Holden et al PNAS 2014

*E. coli* 2D PALM

Buss et al PLoS Genetics 2015

More on this next time...
Chemotaxis sensors

Tar proteins senses chemicals outside of cell
Large clusters of Tar act cooperatively to amplify signals
How are clusters organised?
Chemotaxis sensors

Continuously varying distribution of cluster sizes
→ Suggests stochastic nucleation (ie no defined cluster size)
→ Potential explanation for spontaneous polar clusters

Structured illumination microscopy: principle

It’s kind of subtle...
Let’s discuss it with the ukulele...

Moire fringes project high frequency information (invisible) to lower frequency

Example in practice:
http://zeiss-campus.magnet.fsu.edu/tutorials/superresolution/hrsim/indexflash.html

Related techniques: iSIM, Airyscanning
Resolution

**Spatial resolution**

<table>
<thead>
<tr>
<th>XY</th>
<th>115 nm</th>
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<tbody>
<tr>
<td>Z</td>
<td>350 nm</td>
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**Time (typ.):** 0.6 - 1 s

**Advantages:**
- FAST!
- Low laser power
  - Low phototoxicity
  - Extended time lapse
- Really good at multicolour

**Disadvantages:**
- Lower resolution

*E. coli* RecA

Lesterlin et al. Nature 2014
SpolIIE DNA pump recruitment to *B. subtilis* septation sites

SpolIIE is a translocase – pumps chromosome into forespore
Directly visualized localization to leading edge of closing septum

Fiche et al PLoS Biol 2013
Stimulated Emission Depletion microscopy

Resolution: 50 nm. Time resolution: 1-2s

My view:
- Serious problems with photobleaching make this unsuited to live cell imaging...
- STORM is better at fixed cell
Single molecule imaging

• Closely related to localization microscopy

• Key techniques
  • Single molecule tracking
  • Molecule counting
One of the earliest really powerful applications of single molecule imaging Proteins are expressed and observed in real time Direct observation of “bursty” expression – ie. multiple protein expressed rapidly after transcription of a single mRNA
These days normally combined with photoactivation to obtain 1000s of tracks
→ Single particle tracking PALM (sptPALM) – extremely powerful in bacteria

Can study the binding/ diffusion of all the copies of a labelled protein in a cell
Single molecule tracking of E. coli DNA polymerase I

DNAP I is a repair polymerase
Track its motion –
- Fast diffusion – DNA unbound
- Slow diffusion – DNA bound
Direct observation of DNAPs actively repairing DNA gaps & nicks
- Repair times
- Search times
Single molecule counting by photobleaching

Watch foci bleach step-by-step
→ Tells you how many proteins are in the focus

Reyes-Lamothe et al, Science 2010
Single molecule counting by photobleaching

Very cool paper
By measuring numbers of all the key replisome proteins, determined in vivo stoichiometry of replisome
They found an extra polymerase!

Reyes-Lamothe et al, Science 2010
Single molecule counting by localization microscopy

Since you localize the molecules one-by-one, why not count them?

Potentially very powerful for large complexes where photobleaching would not work
BUT - determining absolute numbers (rather than relative stoichiometry) is an ongoing challenge - mainly due to difficulty establishing ‘dark’ fraction of FPs
Need good “counting standards”

Endesfelder et al, Biophys J 2012
Summary

• Single molecule imaging and super-resolution are powerful tools for both bacterial cell biology and in vivo biophysics
• Rapidly moving, exciting area

• Biology, one molecule at a time!
References


