Tips and tricks for PALM

Seamus Holden
Bacterial Biophysics Lab
Newcastle University
My basic advice

- Do not do STORM/ PALM as a side project!
- Be prepared to commit 1-2 years of your life to it - or find an expert collaborator

- It’s not like “turbo confocal”
- Single molecule imaging requires you to become a specialist

- In the right hands, it is a spectacularly powerful technique
- But it’s easy to screw up in subtle and crucial ways
The diffraction limit

A. Diffraction limits the resolution of light microscopy

B. Sizes of various biological entities and the diffraction limit

Huang et al, Cell, 2010
Localization microscopy: imaging a point source

Fluorescent protein (3 nm)
Localization microscopy

A. Normal Fluorescence Image (FPALM: All Molecules at Once)

B. All Molecules Turned Off (Inactive)

C. Subset 1: Fluorescent Molecule

D. Photobleach

E. All Molecules Turned Off

F. All Molecules Turned Off

G. Subset 2

H. Photobleach

I. All Molecules Turned Off

J. 20 Molecules Localized

K. 40 Molecules Localized

L. 120 Molecules Localized

M. 7516 Molecules Localized
STORM over the Eiffel Tower

Henriques et al., Nat. Methods (2010)
It’s all about making fluorophores blink!

Switchable organic dyes:

Cy5

Heilemann et al., Angew. Chemie, 2009
It’s all about making fluorophores blink!

Switchable fluorescent proteins:

Photoconvertible: eg, mEos2

Photoactivatable: eg, PAmCherry, Dendra2

McKinney et al, Nat Methods 2009
Subach et al., JACS 2010
Localization microscopy

5-20 nm resolution
10-50x improvement!

Note on acronyms:
(F)PALM, (d)STORM, GSDIM, PALMIRA, (u)PAINT, BALM, SMACM, SPDM, etc...
All essentially the same technique (with minor variations, if any)

\[ \sigma_x \propto \frac{1}{\sqrt{N}} \]

Huang et al., Nat Methods 2008
PALM v STORM

• Essentially the same technique
• Often:
  • STORM = organic dye + antibody
  • PALM = genetic fusion + photoactivatable fluorescent protein
  • Then PAINT and all the rest...

• Here:
  • Focus on fluorescent protein super-resolution
  • And data analysis

Hess et al., Biophys. J. (2006)
Fusion protein super-resolution

Advantages
• **Live cells**
• Complete 1:1 labelling possible (bacteria, yeast, now higher eukaryotes with CRISPR)
• No permeabilization, staining → less perturbative (if your fusion is good)
• High labelling specificity
• High labelling density
• Single molecule counting
• Single molecule tracking

Disadvantages
• Low photon count → lower resolution
• Protein fusions can be perturbative
• Multicolour very challenging
First you need a sample

- Many photoactivatable/ photoconvertible fluorescent proteins
  - Photoconvertible often easier to use than photoactivatable – can see the structure!

The really important thing no one tells you about:
- **Protein fusion toxicity/ functionality**
- Ill characterized but probably due to FP-induced aggregation
- **My most reliable PALM FPs:**
  - Dendra2, mMaple3, mEos3.2
- Codon optimize
- Single copy fusion under native protomotor best
- Overexpression of exogenous fusion can be very misleading:

![FtsE-PAmCherry](image)
(It’s supposed to be a ring!!)
Then you need a microscope...

Excellent commercial STORM/ PALM offerings:

• Nikon
• Zeiss
• Leika
• Vutara
• Oxford Nano Imager
• GE-Deltavision

Or you can build it yourself:

More flexible
Cheaper

But do not underestimate the time & challenge!

€ 20k STORM/ PALM microscope
PALM in fixed cells

- Simplest, usually best approach
- No concerns about cell movement or phototoxicity
Fixation method is critical

- Harsh fixation prevents photoactivation of FPs

- STORM example: poor fixation perturbs cytoskeleton

- Carefully optimize your fixation conditions
  - Starting points:
    - Eukaryotes: 2% PFA, 1x PHEM, 15 min
    - Bacteria: 1% PFA 1x RT, 5 min

Endesfelder et al. Biophys J (2013)
Leyton-Puig, Bio Open (2016)
Sample preparation

With single molecule sensitivity, even single molecules of dirt become a problem!

Slide cleaning options:
• Plasma cleaning
• Acid washing

Use 170 µm thick #1.5 slides!
Easiest to use 25mm diameter glass slides in one of these slide holders:
(commercial Attofluor cell chamber works well)

Bare slide, pre plasma cleaning  Post plasma cleaning

http://blog.everydayscientist.com/?p=3296
Dempsey et al Nat Meth 2011
Drift correction - use fiducials if possible

Drift is bad:

Cross correlation works on many structures but lower resolution

Fiducials:

- 100 nm gold nanoparticles work well (eg Corpuscular Inc)
- Fluorescent nanodiamonds were recently used for < 1nm correction – they sound cool!
Bacterial sample preparation tips

• Use low speed centrifugation (1000g) post-fixation if you don’t want burst cells!
• Agarose pads strongly preferred to polylysine – better immobilization.
  • Good protocol: Jong et al, JoVE (2011)
  • Don’t forget fiducials though – agarose contraction!

• Low autofluorescence agarose is important:
  • Invitrogen Ultrapure Agarose works
Initial characterization is crucial

Always have a negative and positive imaging control in initial experiments

Un-labelled cells – control for background/ autofluorescence (bad conditions here)

Positive control – microscope and sample prep QC

Your favourite strain. Eg here
Caulobacter FtsZ-Dendra2
Data acquisition tips

Illumination: TIRF or near-TIRF for low background
Respect imaging density:
Overlapping molecules kill resolution!

Stay in the linear range of your camera
Careful your filters very carefully! – more photons - less background
• Single colour filters best
Image processing

There is a lot of software
How to choose?
- Localization Microscopy Challenge is a great place to start

Simulated datasets to compare software.
2D challenge: Published
3D challenge: (still writing the manuscript)

Good go-to software point: ThunderSTORM (winner in several classes)

http://bigwww.epfl.ch/smlm
Sage et al. Nat Methods 2015
https://github.com/zitmen/thunderstorm
2D data visualization

You start with a list of points – how to make a picture?
• Usually: blur the points with the localization precision
• ThunderSTORM & PALMsiever are user friendly ways of doing this
3D data visualization

Harder to render points in 3D
VisP (Beheiry & Dahan, Nat Meth (2013)) does an excellent job:

Bacterial cell membrane
Data analysis

Can we go beyond pictures?
Clustering, counting, colocalization
Yes – with good controls & careful analysis
Controls: counting standards

Some user friendly software is becoming available, eg LAMA:

Counting standard papers
Resolution depends on sampling

The fewer molecules you image the lower resolution your picture:

Resolution can be estimated by Fourier Ring Correlation:

Nieuwenhuizen et al Nat Meth (2013)
Routes to two colour

Green switchable FPs suck!
YFP + (mCherry or Dendra2) works ok

- Use a 514 laser (lower autofluor)
- You will only see 20% of YFP

Multi-colour protein organization
Routes to two colour

Recent cell-permeable JF dyes are a really exciting development!

Htt84Q–mEos3.2 + H2B-HaloTag-PAJF646

Game changing tech:
- Organic dyes
- 1:1 labelling
- Live cells
- Fixation resistant

Grimm et al Nat Meth (2016)
Localization microscopy in live cells – powerful, but be very careful

- Works – and can be extremely informative
- Eg – here – patchy bacterial cytoskeleton
- But – SLOW!
- >20 s per image
- And be very careful about phototoxicity

- Speed up by HD fitting (DAOSTORM etc) helps but reduces resolution

Caulobacter crescentus FtsZ

Holden et al PNAS (2014)
Holden et al, Nat Meth (2011)
Localization microscopy movies – don’t!

- Not robust due to stochastic image formation and limited # of molecules
- No literature demonstrations beyond initial proof of concept
- Phototoxicity seriously problematic
- SIM usually a better bet

Artefactual dynamics are a big problem
Case in point – FtsZ “dynamics”
Live cell single particle tracking PALM
(the coolest bit of PALM!)

- Use photoactivation to track the entire protein population in a cell!
- Limitation: short track lengths

AMPA membrane interactions in dendritic spines

E coli DNAP response to DNA damage

Manley et al Nat Meth (2008)
Hoze et al PNAS (2012)
Uphoff et al, PNAS (2016)
Live cell single particle tracking PALM

- Again – JF-dyes are extremely promising here – can track for many seconds!

Grimm et al Nat Meth (2016)
Good reviews/ guides

Super-resolution introduction
• **** Navigating challenges in the application of superresolution microscopy, Lambert & Waters, JCB (2016)

PALM detailed protocols

Fixation artefacts
• PFA fixation enables artifact-free super-resolution imaging of the actin cytoskeleton and associated proteins, Leyton-Puig, Bio Open (2016)

Phototoxicity:

Specialist guides:
• Single-Molecule and Superresolution Imaging in Live Bacteria Cells, Biteen & Moerner, CSHL Persp Biol (2010)
A word of advice

• Getting bad, spotty, STORM/ PALM images is easy
• Obtaining beautiful images like this is hard, but worth it!

Don’t settle for poor quality data!

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Good luck!
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seamus.holden@ncl.ac.uk

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