Quantitative analysis of single molecule tracking in live cells: from Brownian motion to function

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"Progress in science depends on new techniques, new discoveries and new ideas, probably in that order." Sydney Brenner
This lecture

- Crash course on optical microscopy
- Browse through the single molecule localization techniques
- Single molecule analysis of the mechanism of neurosecretion (exocytosis)
Super resolution microscopy

Separation of fluorophores:
- ~500 nm
- ~250 nm
- <250 nm

Appearance under optical microscope:
- Resolved
- Rayleigh criterion
- Unresolved
Fluorescence microscopy

Fluorescence microscopy allows visualisation of the distributions of fluorescently-labelled molecules within a sample in a relatively non-invasive and specific way in cells or tissues.

One of the major limitation is the resolution limit set by the **diffraction of light**, which restricts the amount of information that can be captured with standard objectives. This is often referred to as the diffraction barrier, which restricts the ability of optical instruments to distinguish between two objects separated by a lateral distance less than approximately half the wavelength of light used to image the specimen. Basically: two nearby molecules = 1 fluorescent blob...

**Super-resolution techniques**: in the past few years, a number of novel approaches have been employed to circumvent the diffraction limit, including near-field scanning optical microscopy (NSOM), stimulated emission depletion microscopy (STED), stochastic optical reconstruction microscopy (STORM) and structured illumination microscopy (SIM). These techniques have all achieved improved lateral (x-y) resolution down to tens of nanometers, more than an order of magnitude beneath that imposed by the diffraction limit, but each method has a unique set of limitations.
Single-molecule localization microscopy (SMLM) bypasses the lateral resolution limit by separating the fluorescence emitters in time so that they appear one after another stochastically in locations that are sufficiently far apart for their accurate localization. For this purpose, photoactivatable or photoswitchable fluorophores are used, thus enabling the reconstruction of high-resolution images.

Typically, SMLM experiments are either performed in a wide-field configuration, or in total internal reflection (TIR). The advantage of TIR fluorescence microscopy is that the excitation of fluorophores is restricted to about 100 nm along the axial plane and thus reduces the observed axial depth. Two concepts that rely on photoswitching or photoactivation are direct stochastic optical reconstruction microscopy (dSTORM) and photoactivated localization microscopy (PALM).
dSTORM

The key of SMLM is to limit the number of fluorophores detected at a given time to allow accurate localization of single-molecules individually. In the dSTORM approach, this is achieved by operating conventional synthetic fluorophores (i.e. commercially available, for example as antibody conjugates) as photoswitches in the presence of reducing buffers.

dSTORM exploits the fact that most fluorophores are prone to reduction that promote transit into a long-lived, non-fluorescent radical or other reduced state (eg triplet state). The transition into this “dark” state is governed by the intensity of the excitation light (typically laser induced), and the reducing potential of the buffer reagents (typically thiols such as mercaptoethylamine).

The back-transition (or return into the “bright” (or fluorescent) state is governed by residual oxygen, the thermal stability of the off-state and the intensity emitted fluorescence (typically blue-shifted to about 100 nm to the excitation light). The dSTORM concept allows reversible photoswitching of many commercially available fluorophores such as are Alexa Fluor® 647, Alexa Fluor® 532, ATTO 647N etc.
temporal separation of nearby fluorophores. Fitting of point-spread functions of single emitters allows accurate position determination. Super-resolution images can then be reconstructed.
Lippincott-Schwartz, J. and Manley, S.

*Putting super-resolution fluorescence microscopy to work.* Nature Methods 6: 21-23 (2009). A nice overview of the potential benefits and pitfalls of superresolution imaging with emphasis on PALM and related single-molecule techniques. The authors suggest a set of guidelines for presenting images and point out inconsistencies in the current literature.
Nanoscale dynamic organisation of exocytic molecules
Li et al., TiBC in press
Low-resolution solution structures of Munc18:Syntaxin protein complexes indicate an open binding mode driven by the Syntaxin N-peptide

Munc18-1$^{\Delta317-333}$ unable to rescue neuroexocytosis in DKD-PC12 cells
sptPalm

Activation

Imaging

Tracking

DKD cells + Munc18-1-mEos

20Hz imaging + tracking with PALM-tracer on Metamorph

Kasula et al., 2016, Journal of Cell Biology
sptPALM

Munc18-1$^{\text{WT}}$ mEos

Kasula et al., 2016, Journal of Cell Biology
PALM autocorrelation analysis
Munc18-1\textsuperscript{WT}

- g(r) vs. r (nm)
- Unstimulated vs. Stimulated
- Cluster radius (nm)
- Localizations/cluster

** Statistical significance
Munc18-1$^{317-333}$
Munc18-1^{WT}  Munc18-1^{Δ317-333}
Effect of Munc18-1 on Syntaxin-1 nanoscale organisation

Kasula et al., 2016, Journal of Cell Biology
Munc18-1<sup>WT</sup> + Sx1

Munc18-1<sup>Δ317-333</sup> + Sx1

Munc18-1<sup>WT</sup> + Sx1

BoNT/E

Munc18-1<sup>WT</sup> + Sx1

TeTx

Kasula et al., 2016, Journal of Cell Biology
Munc18-1 domain 3a hinge loop controls the opening of Sx1 and its engagement into the SNARE complex.

- Syntaxin-1 nanoclusters are controlled by NSF and α-SNAP and the PIP2/PIP3 binding domain KARRA of Sx1A (Bademosi et al., *Nature Communications*, 2017) and... general anaesthetics (Bademosi et al., *Cell Reports*, 2018).
Opened bunch hypothesis

French kiss hypothesis
We identified two residues that may mediate Munc18-1 and VAMP2 interaction, closely resembling Vps33 and Vam3 interaction in yeast. (Baker et al. 2015)

Using this we cloned Munc18-1 A297 and T304 residues with Histidine to create steric hindrance and affect its binding with VAMP2
Activity-dependent release of Munc18-1 from nanodomains

Increase of Munc18-1 mobility due to its release from nanodomain confinement in DKO-PC12 cells

In agreement with our previous findings in Kasula et al., 2016.
Munc18-1 binding to VAMP2 underpins an activity-dependent release of Munc18-1

Mobility of VAMP2 binding deficient Munc18-1 mutants does not increase after stimulation.

Unpublished data
Syntaxin-1A-GFP (uPAINT) + Munc18-1$^{WT}$-mcherry
Syntaxin-1A + Munc18-1A297H

Syntaxin-1A+Munc18-1A297H

MSD (μm²)

Time (s)

Frequency distribution (fractions)

Log (D)

Mobile fraction

Syntaxin-1A + Munc18-1A297H
Syntaxin-1A + Munc18-1<sub>T304H</sub>

- MSD (µm²) vs. Time (s)
- Frequency distribution (fractions) vs. Log (D)
- Mobile fraction

**Graphs:**
- MSD vs. Time (s) showing a linear increase.
- Frequency distribution (fractions) vs. Log (D) with a peak at log(D) = 0.
- Mobile fraction data points with error bars.
Conclusions

Munc18-1\textsuperscript{WT} undergoes an activity-dependent conformational change leading to Sx1 opening and engagement in the SNARE complex.

Munc18-1 domain 3A extended conformation is probably favoured by VAMP2 binding leading to Syntaxin1A opening. Syntaxin-1A opening therefore occurs within the context of vesicular docking (French kiss hypothesis) which may favour proper templating of the SNARE complex during assembly.
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