Localisation Microscopy Immunolabelling Guide

Aim of guide –

The intention is to highlight additional labelling considerations for localisation microscopy (dSTORM/STORM/GSDIM) and how they affect super-resolution image quality. Many of the principles also apply to other affinity binding approaches such as fluorescent ligand-receptor reagents, e.g. EGF-EGFR, and phalloidin staining of actin. For the sake of brevity, this guide will refer to STORM and antibodies, although most of the guide should be applicable to other localisation microscopy and immunolabelling and affinity binding approaches. The final page contains some references to published protocols. This list is not comprehensive and is intended to be a starting point from which to work. These protocols will not necessarily be ideal for your experimental systems.

This guide assumes that you are already familiar with fluorescence microscopy and immunolabelling approaches. STORM imaging requires high contrast bright fluorophore signal against a minimal background. The better this contrast, the better the resolution. So whilst autofluorescence is still a problem it will not manifest itself directly in the final image. Non-specific labelling, i.e. where there is antibody-fluorophore bound to the sample, but not specifically to the epitope, will still be imaged by STORM. Image filtering based on intensity and or noise may not be a good way to remove this signal as specific labelling at high resolution will also be removed.

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Fixation, Permeabilisation, Quenching & Blocking

Fixation

Some antibodies only bind when their target epitope has been fixed with particular protocols. Some fixation protocols can cause autofluorescence or other artefacts. Also, fixation can cause some types of fine cellular substructural details to be lost, for example fine tubules. These sorts of fine structures are more easily resolvable with STORM, so this may need to be considered when interpreting images.

Permeabilisation

Required for disrupting membranes which allows antibody access to internal cellular structures. Therefore permeabilisation may affect high resolution membrane imaging results, if for example you have used lipid or integral membrane labelling approaches.

Quenching

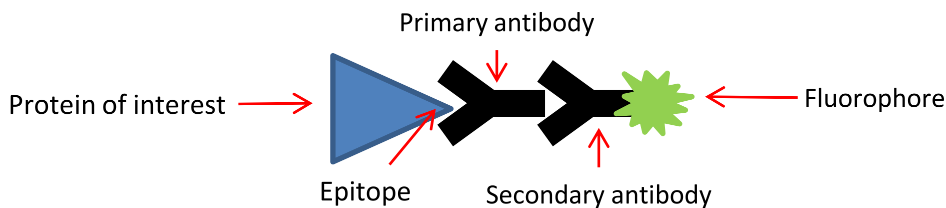
Reduces autofluorescence in the sample leading to improved contrast. This may help to increase the number of localisations and the resolution in resulting super-resolution images.

Blocking

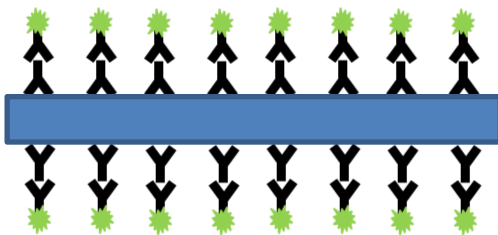
Reduces the amount of non-specific binding of the antibody to the sample. Antibodies should only be bound to their specific epitopes; however, it is possible for lower affinity non-specific binding to occur. This will usually be a relatively low abundancy compared with specific labelling; however, it will be imaged by STORM with high resolution. This may be more of an issue in STORM images as low density areas are usually displayed with relatively high contrast compared with traditional fluorescence imaging techniques.

Label Size

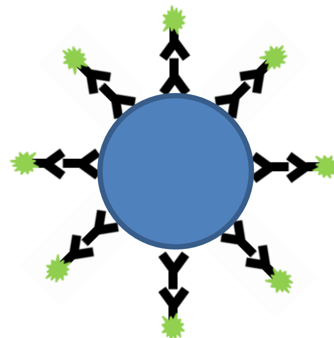
Effective resolution can be improved by reducing the distance between the epitope and the fluorophore. As much as 20 nm of distance can be introduced when using primary and secondary antibody approaches:



With a diffraction-limited resolution of 200 nm this labelling distance may only comprise 10% of the observed "blurring" whereas with localisation microscopy techniques providing up to 20 nm resolution, this becomes far more significant. Labelling distance should be considered when planning experiments and then analysing and interpreting super-resolution images. It may be worth considering approaches that reduced label size such as directly conjugating fluorophore to the primary antibody. Alternatives to traditional antibody labelling include, Fab fragments, nanobodies, aptamers and genetically encoded tags such as SNAP and HaLo.



This filament with a diameter of 10 nm and 15 nm additional labelling distance would end up with an apparent diameter of 40 nm



This vesicle with a diameter of 50 nm and 15 nm additional labelling distance would end up with an apparent diameter of 80 nm

Labelling Density

Incubation times

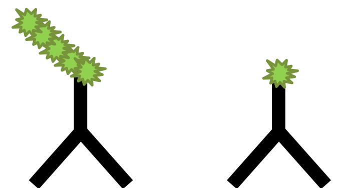
Typical incubation times are 30 to 60 minutes at room temperature. Longer incubation times will increase the chances of both specific and non-specific binding. High affinity specific binding (to the protein of interest) will occur more quickly than low affinity non-specific binding (to other cell structures and the cover glass). Optimum incubation times may vary, depending on the antibody and labelling conditions, such as blocking.

Antibody concentration

Normally the aim should be to use a concentration of antibody that just saturates the available binding sites, i.e. all of the proteins of interest in the sample get tagged with antibody. The resulting super-resolution image will hopefully then report on the positions of a sufficient number of these molecules to be representative. Increasing the concentration beyond saturating is likely to lead to non-specific binding, which should be avoided. Therefore it is recommended to do titrations of antibody concentrations. Commercial secondary antibodies should normally be used in a range of 1:500 to 1:1000 dilution. Polyclonal antibodies will usually lead to higher specific labelling densities than monoclonals. They may also be more prone to non-specific labelling artefacts.

Degree of labelling

Commercial fluorescent reagents, such as secondary antibodies, usually have more than 1 fluorophore molecule per antibody. Typically they are conjugated so that there are 3-8 fluorophore molecules per antibody. This information can usually be found on the product tube or data sheet and may be expressed as DoL or as moles. Ideally for localisation microscopy this ratio should be 1:1. There are a number of protocols and references which describe how to perform custom fluorophore-antibody conjugations.



Left: DoL of 5 (5 dyes per antibody)

Right: DoL of 1 (1 dye per antibody)

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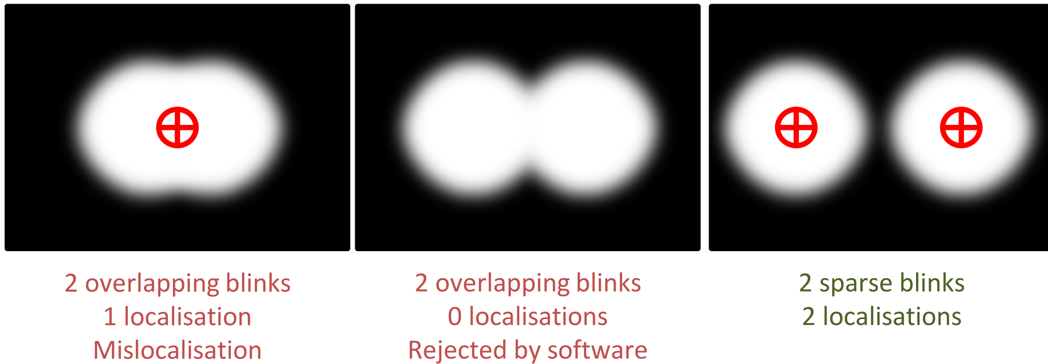
Blinking density (high density problem)

When performing localisation microscopy with an algorithm that is fitting single molecule positions, only one fluorophore molecule should be “switched on” within a diffraction limited area in a given frame. In other words, there should be 200-300 nm space between neighbouring simultaneous “blinks”. The more dense the number of fluorophore molecules the higher the chance there is of having overlapping blinks (non-sparse) data. Possible outcomes where there are 2 simultaneous blinks in close proximity when using a single fitting algorithm:



2 overlapping blinks
1 localisation
Mislocalisation

2 overlapping blinks
0 localisations
Rejected by software

2 sparse blinks
2 localisations

The density of the fluorophore molecules is dependent on:

- Labelling saturation (proportion of molecules of interest bound with antibody) - Ideally saturated unless the underlying structure is very dense. Reducing incubation times, label concentration or mixing with unlabelled antibodies may help to reduce density.
- Density of underlying structure of interest - If it is very dense it may be necessary to use a sub-saturating labelling strategy
- Degree of labelling (fluorophore-antibody ratio) - Ideally 1

The probability of an overlapping blink is dependent on:

- The density of the fluorophore molecules (see above)
- The buffer conditions (fluorophore environment)
- The acquisition settings (eg. exposure time)
- Illumination conditions (eg. laser power, illumination angle)
- The fluorophore (different fluorophores have different chemistries)

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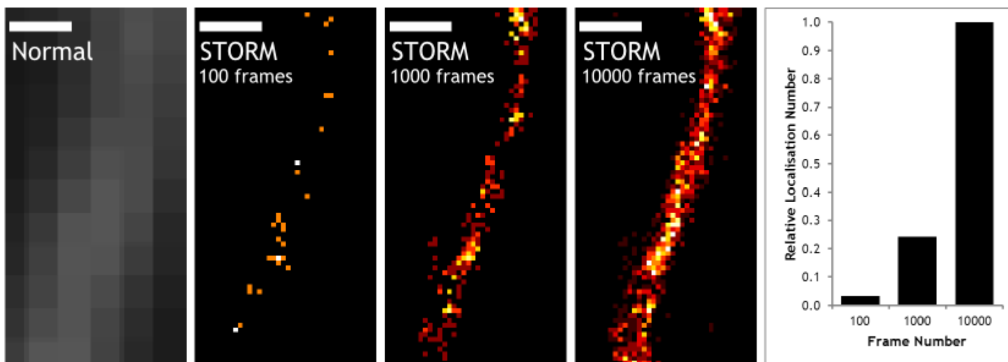
Sparse labelling (low density problem)

The fluorescent labelling density must be at least twice the intended resolution of the image, ie. similar to the localisation precision. For example if you are imaging a structure with 40 nm lateral resolution (approximately 20 nm localisation precision) you will need a label spacing of 20 nm or less. If the labelling density is lower than this the resulting images will appear “pointillist” (punctate):



▲ Fluorescently labelled actin filament (Phalloidin-Alexa 647). Scale bars are 200 nm. STORM pixel size is 15 nm. Normal pixel size is 100 nm. Graph and STORM images show effect of increasing frame number used to reconstruct final image.

Low density labelling (sub-saturating concentrations) may occur if the antibody concentrations is too low, the incubation time is too short, inappropriate fixation conditions are used that destroy the epitope or if the epitope on the protein of interest is masked by other proteins.

In addition to sample preparation problems, pointillist (punctate) images can also be a result of problems with:

- Dye choice - poor characteristics for STORM
- Buffer conditions – wrong buffer choice or problem with buffer
- Illumination conditions (laser power and illumination angle)
- Camera settings (exposure time, frame number)
- Image reconstruction – inappropriate algorithm settings and thresholds
- Visualising the data with a scatter plot method or very small pixels

Fluorophore Choice

Fluorophore selection

In practise the behaviour of the fluorophores depends on the illumination conditions and the chemical environment, ie. buffer. The optimum conditions may vary between fluorophores. Characteristics to consider include:

- Brightness – the more photons emitted per fluorophore "blink" the better the resolution.
- Duty cycle – the proportion of time a fluorophore spends in the "on" state. Therefore on dense samples it is necessary to have as low a number as possible to ensure sparse, non-overlapping blinks. On low density samples this can be higher. In other words, the duty cycle number determines the maximum sample labelling density before overlapping blinks become a consideration.

Example photoswitchable dye combinations

Far red - Cy5 or Alexa 647
Red - Alexa 555 or Alexa 568 or Cy3B
Green - Atto 488

All of these fluorophores photoswitch in standard oxygen scavenging buffers, such as glucose oxidase with 100 mM MEA. They can all be illuminated with an appropriate single laser line which can create the blinking signal required for STORM, for example Alexa 568 with a 561 nm laser. The best resolutions have been achieved with Cy5 and Alexa 647. Green dyes tend not to perform as well as red and far red dyes. Other fluorophore combinations are possible, however, they may not offer the same resolution or work in the same buffer conditions.

For more details on photoswitchable dye performance see Dempsey et al., Nature Methods, 2012.

In addition to using reversibly switchable dyes (as above) it's also possible to use probes (dyes and fluorescent proteins) which can be activated and inactivated using photoactivation strategies of illuminating with 2 or more wavelengths of light to generate sparse blinking signals.

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Dyes, fluorescent proteins & photochemistry

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Alternative labelling strategies

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This list is intended to provide some examples of labelling strategies and is not intended to be comprehensive.

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