doi:10.1017/S1431927614012446
Microsc. Microanal. 20 (Suppl 3), 2014
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Practical Considerations for Single Molecule Localization Microscopy Sample Preparation

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Superresolution fluorescence microscopy is a term used to describe a suite of far-field methods where image resolution is decoupled from the limit imposed by the diffraction of light. Of these methods, one of the most powerful and popular is single molecule localization microscopy (SMLM), including STORM [1], PALM [2], and related methods. These techniques are predicated upon the identification and localization of individual fluorophores to a sub-diffraction limited area. Fluorophores are separated both spatially and temporally by utilizing probe-specific photo-switching properties.

Though capable of providing an order of magnitude resolution increase in both the lateral and axial dimensions, SMLM is limited to a relatively small pool of effective fluorophores. Additionally, given this dramatic increase in sensitivity, proper sample preparation is a must. Here we will discuss probe selection and other aspects of sample preparation for SMLM.

Many organic synthetic dyes [3] and fluorescent proteins (FPs) have been demonstrated as suitable for SMLM. There are several factors that determine how suitable a given fluorophore is for SMLM. Firstly, the accuracy with which one can localize an individual molecule is inversely proportional to the number of detected photons. Fluorophores should spend the majority of the imaging time in a dark non-fluorescent 'off' state. This is necessary to ensure that the Airy profiles of multiple emitters do not overlap and thus perturb the fitting algorithm, especially if the structure in question is densely labeled. Finally, fluorophores should be capable of repeatedly cycling between the dark and fluorescent states to facilitate multiple localizations.

Inducing photoswitching in dye molecules usually involves a reducing and oxidizing (ROXS) buffer system, with optimal formulations differing between different classes of dyes [4]. To this point, the industry-standard fluorophores are the far-red emitting carbocyanines Alexa Fluor 647 and Cy5, which emit ~3000-6000 photons per emission event. Dyes emitting outside of this spectral range do not perform as well. Notable dyes include the red-emitting Cy3B and Alexa Fluor 568, and the green-emitting ATTO 488 and Alexa Fluor 488. Though considered to by-and-large possess superior properties compared to FPs, synthetic dyes are traditionally difficult to use with live cells, most often confined to immunofluorescent labeling. However, some cell-permeable target-specific dyes have been proven suitable for live-cell SMLM imaging (e.g. MitoTracker Red). A more recent strategy involves using genetically-expressed bio-orthogonal small molecule tags, such as SNAP, CLIP, HALO, TMP, and others.

Though not as bright as synthetic dyes, FPs hold the advantage of being genetically expressed in the host system. Furthermore they are often desirable for quantitative superresolution imaging; a 1:1 relationship exists between each FP and its fusion partner, additionally most FPs only undergo a single emission event. FP photoswitching comes in several varieties: they can be reversibly photoswitched

https://doi.org/10.1017/S1431927614012446 Published online by Cambridge University Press

between a dark and fluorescent state, permanently photo-activated from a dark to fluorescent state, or have their emission bandwidth permanently red-shifted upon photoactivation.
By-and-large sample preparation for SMLM is the same as for conventional widefield fluorescence microscopy, but with an increased emphasis on structural preservation, autofluorescence reduction, and minimizing non-specific staining. The sensitivity afforded by such a high resolution technique demands the lowest background and greatest structural preservation possible. Fixation-induced autofluorescence should be mitigated. If using paraformaldehyde, follow fixation by washing with PBS with an added 50 mM glycine; the glycine reacts with free aldehyde groups, stopping fixation and helping to quench fixation-induced autofluorescence. Glutaraldehyde is much more reactive (having two aldehyde groups per molecule), following fixation the sample should be washed using a 0.1% (w/v) solution of sodium borohydride in PBS to reduce free aldehyde groups.

Several other experiment-specific factors must be considered, including choice of culture vessel/coverglass, substrate coating, labeling system (including antibodies), identification of compatible fluorophores for multi-color and live-cell imaging, and more. Each of these topics will be discussed and example protocols given for labeling a variety of cellular structures for SMLM imaging.

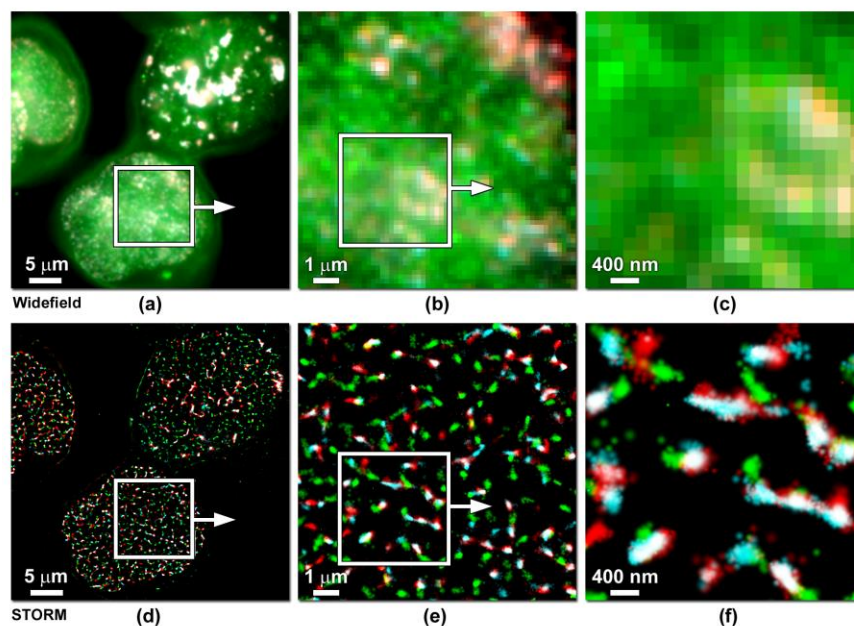

Figure 1. Three-color SMLM image of Alexa647-EdU (red), Alexa568-PCNA (cyan), and ATTO488-MCM3 (green) in S-phase CHO cells. (a-c) Widefield images of S-phase nuclei, zooming in as marked by the ROIs from left to right. (d-f) SMLM images corresponding to the widefield images (a-c), respectively. Image reproduced from [5].

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