map is typically an order of magnitude better resolved, because it is generated from fluorophore positions determined with high (~10-30 nm) precision.

To correctly interpret images of unknown or little-understood specimens – which is precisely the task for which super resolution imaging is most important – it is crucial to know the resolution of the system. When we refer to the localisation microscopy system, this includes the behaviour of the data fitting and visualisation algorithms that are applied to measurements of a particular specimen, as well as the optical instrument itself. Unfortunately, the resolution achieved by localisation microscopy can vary greatly between samples, because the accuracy of data fitting and visualisation depends on the quality of fluorescence images and background noise that are collected from the specimen. Therefore it is not sufficient to evaluate the instrument resolution using a calibration sample, and then state this as the resolution when studying other objects. Instead, it is important to estimate the particular resolution of each super-resolution image. This can be done empirically when measuring well-known samples such as actin filaments or sparse dye molecules on a coverslip – and this is often done for system calibration (Xu et al. 2012) – because the reconstructed image can be compared with the known structure. For unknown specimens it is necessary to make a blind assessment of how each localisation microscopy image is limited by its own source data. This paper discusses localisation microscopy as a Density Estimation technique (Parzen 1962, Silverman 1986), and thus identifies a resolution limit based on localisation precision – a quantity for which we can obtain a blind estimate from raw image data. Density Estimation theory also addresses the effect of localisation frequency: the best super-resolution image obtained with only one localisation per fluorophore tends to be less well resolved than one constructed with several detections, because successful visualisation of limited data requires broader smoothing. Other issues that can worsen the effective resolution include too-sparse labelling, fluorescent label width, and motion blur, and these effects can also be quantified.

Methods

Sample preparation

Pre-formed rabbit skeletal muscle actin filaments (Cytoskeleton Inc) were diluted in general actin buffer (Cytoskeleton Inc) to a concentration of 2 μM and incubated with a 1 μl in 200 μl dilution of 6.6 μM phalloidin-Alexa 647 solution (Invitrogen). These were allowed to adhere to LabTek glass chambers for 2 hours prior to being imaged. Before addition of actin filaments, LabTek glass chambers were treated with 2 M glycine for 30 min at 37°C and then coated with 0.05% poly L-lysine solution for 30 min at room temperature.

Hela cells (ATCC, CCL-2) were grown to 80% confluency in LabTek chambers in DMEM (Fisher, VX31966021) containing FBS (Fisher VX10500064). Cells were then starved in DMEM without FBS for 60 min followed by incubation with 1 μg/ml EGF-Alexa 647 (Fisher, VXE35351) in complete culture medium for 30 min at 37°C. Finally cells were fixed with 4% formaldehyde for 10 min and then washed with PBS.

Microscopy

Fluorescence images were taken on an Olympus IX71 inverted widefield microscope, with a 100x, 1.49 NA TIRF objective lens. To induce photoswitching, the LabTek chambers were filled with a "switching buffer" solution: 100 mM mercaptoethylamine (MEA) in phosphate buffered saline (PBS, pH 7.4), together with a glucose-enzyme oxygen scavenger (40 mg/ml glucose, 50 μg/ml glucose oxidase, 1 μg/ml catalase). Samples were illuminated with 642 nm laser light at 2 kW/cm^2. Stacks of 10^4 images were collected at 65 Hz, with 10 ms exposure times.

Image simulation and analysis

Simulated images were generated in MATLAB, after (Thompson et al. 2002). For the single fluorophore simulation, the expected number of photons arriving on each camera pixel was calculated by integrating a Gaussian PSF over the pixel, and allocating the corresponding proportion of N detected photons. To simulate quantum photon counting, this number was replaced with a random number generated from a Poisson distribution with the same mean. Independent, Gaussian random noise of standard deviation b was then added to each pixel. To simulate a structured object (lines of fluorophores at 5 nm spacing, crossing at 45°), a subset of the fluorophores was randomly "activated" for each camera frame, and the cumulative image of all the active fluorophores was generated as above. Gaussian noise was added at the end of this process.

Super-resolution images were reconstructed using a "Segmentation and Sparse Gaussian Fitting" algorithm, similar to (Wolter et al. 2010), implemented in MATLAB. In each camera frame, local maxima brighter than a threshold value were fit to a Gaussian PSF, by an iterative least squares method. For each spot, a localised position was obtained, as well as an estimate for the number of photons of fluorescence, N. The camera noise b was estimated on a framewise basis as the standard deviation of camera pixel brightness in regions without any bright spots. Average localisation precision was evaluated from N and b using Equation 1. Localised fluorophore density was visualised as a "histogram raster," with simulations