Supplementary information, Data S1 Methods and Notes

Cell culture, transfection and fixation

U2OS cells in McCoy's 5A Medium Modified (MCMM) (Life Technologies), HeLa cells and COS7 cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with glucose (Life Technologies) and 10% fetal bovine serum (Life Technologies) and penicillin/streptomycin (Hyclone) were grown at 37°C with 5% CO2. For transient expression, cells cultured in 12-well plates (Nunc) at 80% confluence were transfected with 1 μg lifeact-mEos3.2 [1] plasmid (from our lab), mEos3.2-CLC (clathrin light chain) [2] plasmid (gift from Prof. Joerg Bewersdorf) or mEos3.2-Mito [1] plasmid (from our lab) using Lipofectamine 2000 (Life Technologies) following the manufacturer's protocol. Five hours post transfection, cells were trypsinized and plated on clean coverslips (Fisher Scientific) coated with 10 mg/ml fibronection (Millipore, FC010) to induce spreading for an additional 24 h. Fixations were performed with PBS buffer (pH 7.4) containing 4% paraformaldehyde and 0.2% glutaraldehyde for 15 min at 37°C just before imaging. During live imaging, cells were incubated in DMEM without phenol.

Sample preparation and dye labelling of ER structure.

Live COS7 cells were stained immediately before imaging as described before [3]. Approximately 48 h after plating, COS7 cells were rinsed with DMEM with glucose and no phenol red (Life Technologies). Then, the cells were incubated in staining solution containing 10 μM ER-Tracker Red (Invitrogen) for 4 min. Imaging buffer was prepared with DMEM supplemented with 2% glucose, 6.7% of 1 M HEPES (pH 7.4), and an oxygen scavenging system (0.5 mg／mL glucose oxidase and 40 μg/mL catalase). COS7 cells were imaged at room temperature.

Imaging system and acquisition

A homemade TIRF microscopy system with an Olympus IX71 body (Olympus) and high-NA oil objectives was used for imaging acquisition. For fixed and live actin imaging, a 100×, 1.49 NA oil objective (Olympus PLAN APO) were used with image pixel size of 160 nm. For live CCP structures, a 150×, 1.45 NA objective and 1.6X intermediate magnifications were used with image pixel size of 66.7 nm. For Mito and ER structures, a 100×, 1.7 NA objective and 1.6X intermediate magnifications were used with image pixel size of 100 nm. The fluorescence signals were acquired using an electron-multiplying charge coupled device (EMCCD) camera (Andor iXon DV-897 BV). For actin, CCP and Mito imaging, a 488 nm laser and a 561 nm laser (Coherent) were simultaneously used to record the green channel signal for Bayesian calculation and the red channel signal for single molecule position extraction. For ER imaging, only the 561 nm laser were used. The maximum power intensity near the back pupil of the objective was 2.5 W/cm^2 for the 488 nm laser and 1.5 kW/cm^2 for the 561 nm laser. During imaging of fixed actin, 20,000 sequential frames were acquired with an exposure time of 50 ms. All 20,000 frames from the red channel

were used to generate the reference PALM reconstruction image as previously described [1], while only the first 200 frames from both channels were used to calculate the SIMBA result. For the live-cell imaging of actin, a shortened 18 ms (whole cell, FOV: 512×256 pixels) exposure time was used to avoid movement artifacts. For every other 100 frames (i.e., a SIMBA image at one time point), a 20 s time break was included for cells to recover fluorescence. For the live-cell imaging of CCP, a continuous 405 nm laser (Coherent) at 2.5 mW/cm^2 near the back pupil was added to enhance the fluorescent signal. 5,000 sequential frames were taken at an exposure time of 20 ms. Drift and dual channel aberrations were assessed and calibrated by monitoring the drift of four color beads (Life Technologies). The exposure time for Mito and ER were 20 ms and 6.7 ms respectively. 200 frames were taken from dual channel without break for Mito reconstruction. For ER reconstruction, 200 frames were first taken with low illumination intensity to produce SIMBA dataset, and then another 200 frames from the same channel were taken with relative high illumination intensity to record the single molecule information.

Single molecule extraction and PALM analysis

PALM analysis were performed using custom routines in MATLAB as described before [1]. For actin, CCP and Mito structures, single molecules were detected by a wavelet transform algorithm with a proper threshold, and molecule locations were determined by finding local maxima with a mask of 5 × 5 pixels. For ER structure, single molecule information were extracted with an open source plug-in for ImageJ, ThunderSTROM [4].

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Supplementary Note

Contents

1. Overall description

The 3B technique models the whole dataset, using information from many overlapping fluorophores, as well as from bleaching events, blinking events and changes by fluorophores being added or removed in the cell [5]. Although the original 3B paper emphasized that common FPs could be used for the 3B microscopy, photo-activatable FPs (PAFPs) offer the advantage of having more "blinking" events and single molecule reappearances. As a result of this, fewer frames are needed using PAFPs for reconstructing a final SR image with sufficient detail to see a structure-of-interest. Indeed, this was illustrated in the original 3B paper: correlative 3B and PALM analysis was better using PAGFP compared to mCherry [5]. PA-mCherry was also shown to be useful for the 3B analysis with cloud computing [6]. With either mCherry or PAFP, all of the fluorophore reappearances in sequential-acquired frames and all the photons collected from a fluorophore are used to determine the position of single molecule by modeling the whole dataset [5]. However, the single fluorophore reappearances cannot be easily viewed by camera during the 3B sampling because of the high labeling density and overlapping of fluorophores.

In 3B calculations, the initial model is set up by choosing random spots, including both the "true signal" and "false signal" single molecules. It is then modified incrementally by adding "true signal" spots and removing "false signal" spots at random positions. After many iterations of this, an optimized model is obtained with more "true signal" spots. This is then used for further model selection. We hypothesize that an initial model with only "true signal" molecules would greatly facilitate the decision of adding a new candidate spot at random positions, and would thus accelerate computing speed and improve spatial-temporal resolution. To accomplish this, we decided to view only a small number of single-molecule reappearances and use these localizations to represent the real position in the whole labeled structure. This information would then be used as the initial model for modeling the whole dataset to obtain higher spatial-temporal resolution and speeding the computing.

Recently we developed a photo-convertible fluorescent protein (PCFP) mEos3.2 [1], which can be photo-converted from green color to red color by 405-nm illumination. In the green state the protein performs very well as a reversibly photoswitchable fluorescent (RSFP) with good on/off properties, so we used it for Bayesian localization microscopy. In the red state, the positions of single molecules were precisely localized by using the single-molecule localization algorithm. After that, the positions of these single molecules were randomly selected as several batches and used in combination with the Quick-3B algorithm to produce different initial model, and then used to constrain the fitting model and look for possible neighbor molecules. Furthermore, we developed an expansion sampling algorithm to further accelerate the computing speed and extend the spatial-temporal resolution. Lastly, we introduced two key techniques, a distance and intensity thresholding method and a modified conjugate gradient algorithm, to reduce the artificial discontinuous dot-shape structures produced by the 3B algorithm.

2. Quick Bayesian analysis of bleaching and blinking (Quick-3B)

Quick-3B is a method that combines a clustering analysis and a modified Bayesian analysis of bleaching and blinking (3B). The clustering analysis segments the whole model into several individual parts based on distance and relevance so that computation time is reduced during model selection and optimization. In this way, parallel computing would be possible. Furthermore, calculation accuracies are expected to be improved due to the isolation of unrelated fluorophores. The 3B method is modified by applying a limited forward algorithm to define relevant small regions instead of the entire image to calculate the observing probability of fluorophores. Quick-3B is seventeen-fold faster than 3B for a field of view (FOV) of 38 × 50 pixels (Supplementary information, Figure S1A). This efficiency further increases as the reconstruction region increases up to 50 × 50 pixels, for which the 3B method requires up to a full day to analyze.

2.1 Clustering analysis

In the 3B method, the determination of the space-time position of one fluorophore is dependent on all other fluorophores. The effects of the other fluorophores on the precision of the calculation of the one fluorophore position are limited by their distance and the size of the point spread function (PSF) of the one fluorophore. If the distance of a fluorophore is far greater than the PSF, the influence from that fluorophore is negligible. The inclusion of very far away fluorophores will add to the computation workload and negatively affect the precision of the calculation. Clustering reduces the impact of the unrelated (i.e., far away) fluorophores and accelerates the computing speed. Clustering groups sets of objects in such a way that objects in the same group are more similar to each other than to those in other groups. We used a k-means algorithm to cluster all fluorophores in the model. For a model with *M* fluorophores, we divided the fluorophores into *k* different clusters and utilized the position information of different fluorophores to decide whether they belonged to the same cluster. This formula is shown in equation 1. The k-means algorithm assigned fluorophores to the closest cluster S_i. When one fluorophore was optimized, we first determined the grouping cluster and subsequently optimized this fluorophore in that cluster. When all fluorophores of a certain cluster have been optimized, we combined the calculations. In this way, the computational workload was greatly reduced.

$$\arg\min_{\mathrm{S}} \sum_{i=1}^{k} \sum_{\mathrm{x}_j \in S_i} \left\| \mathrm{x}_j - u_i \right\|^2 \tag{1}$$

Here, x_j is the position information of the *j*th fluorophore, u_i is the center of the *i*th cluster, and $\left\| \mathrm{x}_j - u_i \right\|^2$ is the distance between the *j*th fluorophore and the cluster center u_i. The expression minimizes the sum of the distances from each fluorophore to its corresponding cluster center.

2.2 Limited forward algorithm

In the model optimization steps, the 3B method uses a hybrid Markov chain Monte Carlo (MCMC) algorithm and a forward algorithm to optimize the parameters of a candidate fluorophore [7]. To obtain the observing probability of each fluorophore (i.e., the probability that a certain fluorophore is within in a frame), the hybrid algorithm is used to calculate the entire image region. Because the computational workload of 3B scales with the product of the number of fluorophores and the number of pixels, the computing time of 3B will dramatically increase as the image size increases. To reduce this workload, fluorophores are only considered if their distance from the central fluorophore is within the region defined by the PSF, which is assumed to obey a Gaussian distribution, $N(u,\sigma)$ (the mean is u and the standard deviation is σ). The intensity of the PSF decays exponentially away from the mean, and 99.7% of values drawn from a Gaussian distribution are within three standard deviations (3σ). Therefore, we proposed a limited forward algorithm to reduce the size of the region for the observing probability. Instead of calculating the observing probability in the entire image region, we only calculate the observing probability in regions within 3σ + 1 from maximum intensity u of a fluorophore. In this way, the computational workload is only scaled to the number of fluorophores multiplied by a constant.

2.3 The Quick-3B algorithm

The steps of a Quick-3B algorithm are as follows:

Step 1. Select initial spot positions for a model as in 3B.

Step 2. Optimize the entire model: re-optimize each fluorophore to obtain a new position in the model.

1) Utilize the k-means algorithm to cluster all fluorophores in the model into different clusters.
2) For one fluorophore in a certain cluster, re-optimize the fluorophore to obtain new fluorophore parameters using the hybrid MCMC and the limited forward algorithms.
3) Merge all fluorophores in different clusters.

Step 3. Model selection: incrementally adjust the model to fit the data, one fluorophore at a time. Either add a new fluorophore at a random position or select a fluorophore in the model for removal.

1) Utilize the k-means algorithm to cluster all fluorophores in the model into different clusters.
2) Either add a new fluorophore or select a new fluorophore at random as in 3B.
3) Determine the cluster the new or selected fluorophore belongs.
4) Optimize this fluorophore in its cluster to adjust the model.

Step 4. Go to *Step 2*.

The repeated execution of *Step 2* and *Step 3* generates a super-resolution image. The algorithm will be terminated when adjacent reconstructed images no longer

significantly differ.

3. Single molecule-guided Bayesian localization microscopy (SIMBA)

The Quick-3B algorithm significantly accelerates the reconstruction speed. However, the algorithm still suffers from artificial discontinuous structures, much like the 3B algorithm (Supplementary information, Figure S1A). To eliminate these artifacts and to expand the spatial-temporal resolution limit of this method, we next introduced a second methodology based on Quick-3B: a single molecule-guided Bayesian localization microscopy (SIMBA). This method makes large-view live cell super-resolution imaging simple and feasible.

In addition to cluster analyses and the limited forward algorithm, SIMBA contains three other key technical aspects. (1) A single molecule localization algorithm accurately extracts the position of a single fluorophore. This information is used as a guide to optimize the Bayesian localization model. (2) An expansion sampling and modified conjugate gradient algorithm utilizes the single molecule information to address the discontinuity caused by "bias" calculations in the Quick-3B algorithm. (3) A distance and intensity threshold removes "false" fluorophores. These three aspects accelerate the computing speed and extend the spatial-temporal resolution of SIMBA.

3.1 Single molecule localization algorithm

SIMBA requires two types of fluorescent signals from the same sample: a single molecule fluorescence and a bulky fluorescence. These signals can be collected simultaneously or successively. We took advantage of a photo-convertible fluorescent protein (PCFP), mEos3.2, which emits a bulky green fluorescent signal under a 488 nm laser and emits a red fluorescent signal at the single molecule level under an intense 561 nm laser alone or with a low intensity 405 nm laser. By simultaneously recording the green and red channel signals of mEos3.2-labelled target molecules, SIMBA was implemented. Briefly, we first used the single-molecule localization algorithm to localize the position of single molecules in the red channel. After calibrating for drift and dual channel aberrations by monitoring the drift of the four color beads, we correlated the red and green channel datasets. The single molecules of the red channel, i.e., the candidate spots, were used as the initial models for the green channel to guide model optimization in SIMBA. Second, based on these candidate spots, we used Bayesian analysis to obtain new fluorophore positions in the green channel. Third, these newly obtained fluorophores, i.e., the expansion spots, were considered new candidate spots for exploring nearby fluorophores. This process was repeated until a final super-resolution image was obtained.

Because the single molecules of the red channel must be correlated with a true position in the green channel, the use of the precise localizations of fluorophores as initial models instead of choosing random positions improved the accuracy of the model. Moreover, SIMBA did not instantaneously consider all positional data. Instead, candidate spots were randomly selected to build the initial model. In the subsequent cycle, new candidate spots were selected from the remaining fluorophores. This

method is equivalent to a "model pool" wherein newly obtained fluorophores are added to the pool to expand the size of the pool. The fluorophores calculated based on SIMBA would be less likely to gather in the same positions. This improved the image continuity. Furthermore, the initial model was directly determined by the "model pool", which simplifies the model selection process and speeds up the computational process.

3.2 Expansion sampling and modified conjugate gradient

SIMBA used a Factorial Hidden Markov Model (FHMM) to model the entire dataset. The parameters of the FHMM were estimated by an expectation maximization (EM) algorithm (equation 2) [8]. However, because the true localizations of a few fluorophores have already been obtained, we modified the EM steps to further utilize the collected data, including expansion sampling to address uneven calculations by obtaining more fluorophores of categorically "less bright" groups and modified conjugate gradient to avoid discrete jumps to local maxima.

$$Q\left(\phi^{\text{new}} \mid \phi\right) = E\left\{\log P\left(\left\{F_t, D_t\right\} \mid \phi^{\text{new}}\right) \mid \phi, \left\{D_t\right\}\right\} \tag{2}$$

Here, the recorded observation data are represented by $\{D_t\}$, where t = 1, …T, i.e., T image frames. The hidden data are represented by $\{F_t\}$. Each fluorophore in the model independently transits among three possible states: emitting (on), non-emitting (off) and bleach. Q is a function of the fluorophore parameters ϕ^{new}, given the current parameter estimate ϕ and the observation sequence $\{D_t\}$.

Expansion sampling: In the E step (sampling), the hybrid MCMC and the limited forward algorithm were used to sample the initial model made up of single molecules. When a new fluorophore was identified, we took samples of this fluorophore using the forward filtering, backward sampling algorithm [9] and added these new samples into the sampling of the initial model. This was repeated to obtain expansion samples. This expansion sampling method enables us to evenly calculate across the entire image region and increases the chance to catch "less bright" fluorophores. Uneven calculations are the main cause of discontinuities, as observed in the 3B and Quick-3B models. Moreover, for each iteration, we only calculated the expansion samples and added these samples to the initial model. Thus, this iterative process reduced the computational workload. Comparatively, the Quick-3B model requires the recalculation of all fluorophores for each step.

Modified conjugate gradient: In the M step (maximizing the expected log likelihood by optimizing the parameters), if the fluorophore parameters were optimized based on the calculated position, newly identified fluorophore would likely jump to the local maximum near the initial position. This would result in artificially "bright" areas. However, calculations of new positions for each identified fluorophore would require more time and computational power. To optimize the accuracy of the

gradient without sacrificing calculation efficiency, we randomly re-selected a new initial position less than two standard deviations (2σ) from the original initial position.

3.3 Distance and intensity threshold

We applied a distance threshold to avoid repetitive calculations of the same position. We used an intensity threshold to balance the intensity distribution of fluorophores.

Distance threshold: If the optimized position of a fluorophore was nearly the same as the pre-optimized position (i.e., distance < 0.01 pixel), this would lead to repetitive calculations and result in localized bright spots. This is especially observed for sparse fluorophore densities. The likelihood that the same fluorophore position will be recalculated would be far larger than other positions in this region because there is too little fluorophores in the surroundings. Therefore, we used a distance threshold to effectively ignore fluorophores when the position distance before and after optimization was less than 0.01 pixel.

Intensity threshold: Because our model started from single molecules and was expanded to include new fluorophores, if a large number of single molecules were identified in a certain region, a higher number of new fluorophores will be expanded there. Because previously identified fluorophores with higher brightness were removed from the model, newer fluorophores will have increasingly lower brightness. Thus, a fluorescent intensity threshold was used to remove "false" fluorophores. The prior distribution was assumed to be a log normal distribution, $log\text{-}N(u,\sigma^2)$ (i.e., the mean is u, and the standard deviation is σ). Here, we set the intensity threshold, $e^{\mu-3\sigma}$, as a lower bound to balance the intensity distribution of the fluorophores.

3.4 The SIMBA algorithm

The detail SIMBA algorithm (Supplementary information, Figure S2) is shown as follows:

Step 1. Pre-processing single molecules from red channel.

a) Extracting single molecules.
b) Selecting the appropriate number of single molecules as candidate spots.
c) Correcting the drift and correlate the red and green channel dataset.

Step 2. Model selection: Candidate spots are randomly selected for the initial model and removed from the candidate spots afterwards. When all candidate spots has been selected once, the expansion spots are assigned as the new candidate spots.

Step 3. Optimizing the initial model:

a) All fluorophores are clustered in the initial model.
b) For each cluster,
    i. The samples of the initial model are obtained using the hybrid MCMC algorithm and the limited forward algorithm.
    ii. For each fluorophore, 4 optimized fluorophores are calculated using the limited forward algorithm and the modified conjugate gradient method.
    iii. Based on the distance and intensity thresholds, a determination is made whether to add the new calculated fluorophores to the expansion spots.

iv. The samples of the new expansion spots are calculated and are added to the initial model by expansion sampling.

Step 4. Go to *Step 2*.

Note that single molecules are processed in the red channel (*Step 1*). We utilized these molecules as candidate spots to gradually expand new fluorophores in the green channel (*Step 2* and *Step 3*). The algorithm was terminated when the adjacent reconstructed images no longer significantly differed.

3.5 Summary

The SIMBA method is a practical tool for analyzing data on a desktop computer and extends the capabilities of the 3B technique for the super-resolution imaging of whole-cell structures over large fields of view for long-term cellular dynamics in living cells. The benefits of SIMBA are summarized as follows:

Regarding accuracy, the single molecule data provides real spatial positions in the corresponding Bayesian calculation channel, which improves the accuracy of the model. The stochastic and renewable properties of the "model pool" ensures model diversity, which improves the image continuity. Additionally, the impact of the unrelated fluorophores were reduced by the introduction of cluster analysis.

The introduction of specific algorithms in SIMBA provides four major benefits. (1) The introduction of localizations of observed single-molecules has three major advantages. First, by using true single molecule localizations in the initial model, it greatly accelerates computing speed by skipping the processes of removing "false signal" single molecules. Second, the positions of single molecules are randomly selected to produce different initial model, which avoids producing artificial discontinuous spot structures resulting from one initial model as in the 3B algorithm. Finally, the positions of single molecules in the expansion sampling algorithm is useful to quickly find the neighbor molecules with high probability, and thus improved the fidelity of SIMBA. (2) Expansion sampling: for each iteration, the sampling region is limited to only new fluorophores. (3) Limited forward algorithm: the calculation area is localized to regions around a single fluorophore PSF. This reduces the computational workload. (4) Clustering analysis: the computation time scale is reduced by considering groups of fluorophores instead of the overall fluorophores.

In a single FOV, improvements in calculation accuracy and speed make it possible to efficiently calculate a whole-cell structure. Moreover, the effects of image segmentation and mosaics can be ignored.

Algorithm-based SR technique SOFI [10] shown the similar advantages of fast and easy implement, however, SIMBA has both higher spatial and temporal resolution as well as less artifact than SOFI. Normally, SOFI need at least 1000 frames to reconstruct an image, which means it has lower temporal resolution than SIMBA. And it usually improves resolution only by two-fold, to ~100 nm. Higher order SOFI analysis may increase spatial resolution, however, it also tend to induce artifacts and degrade the image quality [11].

Similar as PALM/STORM, both the spatial and temporal resolutions of

Bayesian-based methods highly depend on the experimental conditions and the structures to be resolved. For example, shortened imaging time (higher temporal resolution) is necessary for live cell imaging to avoid movement induced artifacts, however, this may also decrease the spatial resolution as compared to fixed cell sample. Therefore, a spatial resolution in a fixed cell sample under prolonged sampling times cannot be readily extended to live cell conditions. Similarly, a structure having lower copies of molecules such as CCPs may have a lower practical spatial resolution than structures with higher copies of molecules, assuming the same temporal resolution. Therefore, a spatial resolution achieved by one sample cannot be readily extended to another sample without verification.

While we used SIMBA to image single molecules and structures near the plasma membrane by employing it with total internal reflection fluorescence (TIRF) microscopy, it should be easy to combine SIMBA with light sheet methods (i.e., lattice light sheet microscopy), which could get rid of the out-of-focus background light, to examine subcellular structures deep within the cells at high resolution. Moreover, employing sCMOS cameras in SIMBA, which can quickly image large field-of-views, should enable even higher spatial-temporal resolutions in 3D.

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