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Autoreferát disertační práce

Výpočetní metody v jednomolekulové lokalizační mikroskopii

Computational methods in single molecule localization microscopy

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

Abstract

Fluorescence microscopy is one of the chief tools used in biomedical research as it is a non invasive, non destructive, and highly specific imaging method. Unfortunately, an optical microscope is a diffraction limited system. Maximum achievable spatial resolution is approximately 250 nm laterally and 500 nm axially. Since most of the structures in cells researchers are interested in are smaller than that, increasing resolution is of prime importance. In recent years, several methods for imaging beyond the diffraction barrier have been developed. One of them is single molecule localization microscopy, a powerful method reported to resolve details as small as 5 nm. This approach to fluorescence microscopy is very computationally intensive. Developing methods to analyze single molecule data and to obtain super-resolution images are the topics of this thesis.

In localization microscopy, a super-resolution image is reconstructed from a long sequence of conventional images of sparsely distributed single photoswitchable molecules that need to be systematically localized with sub-diffraction precision. We designed, implemented, and experimentally verified a set of methods for automated processing, analysis and visualization of data acquired by single molecule localization microscopy and we "packaged" them as an open source software called ThunderSTORM. ThunderSTORM has become one of the top softwares in the field.

Next, we introduce our design for a novel dual-objective super-resolution microscope, which roughly doubles the count of collected photons. This further improves achievable resolution by a factor of $\sqrt{2}$. We built a working prototype of the microscope and developed and

experimentally verified methods for calibration of the microscope and for image analysis.

In the last part of the thesis we address the issue that despite the high spatial resolution of localization microscopy it is not always suitable for live cell imaging due to its limited temporal resolution. One strategy is to increase the density of photoactivated molecules present in each image. Such an approach poses a challenge for the image analysis. We present 3denseSTORM, a new algorithm which is able to recover 2D or 3D super-resolution images from a sequence of diffraction limited images with high densities of photoactivated molecules. The algorithm utilizes methods from compressed sensing and uses a Poisson noise model, which becomes critical in low-light conditions. We derive the theoretical resolution limits of the method and show examples of image reconstructions in 2D and 3D simulations and in real data of biological samples. The method is suitable for fast image acquisition in densely labeled samples and helps facilitate live cell studies with single molecule localization microscopy.

Keywords

Super-resolution microscopy, single-molecule localization microscopy, image processing, numerical optimization, sparse representations.

Abstrakt

Fluorescenční mikroskopie je jedním z hlavních nástrojů biomedicínského výzkumu díky tomu, že se jedná o neinvazivní nedestruktivní a vysoce specifickou zobrazovací metodu. Bohužel optický mikroskop je difrakčně limitovaný systém, což znamená, že nejvyšší dosažitelné rozlišení je přibližně 250 nm laterálně a 500 nm axiálně. Jelikož většina buněčných struktur, o které se výzkumníci zajímají, je menší, zvýšení rozlišovací schopnosti je velice důležité. V posledních letech bylo vyvinuto několik metod, které umožňují zobrazování za hranicí difrakce. Jednou z nich je jednomolekulová lokalizační mikroskopie, která dokáže rozlišit detaily až do 5nm. Tato metoda je však velmi výpočetně náročná. Vývoj metod pro zobrazování a analýzu dat z jednomolekulové lokalizační mikroskopie je předmětem této práce.

V lokalizační mikroskopii je obraz se superrozlišením zrekonstruován z dlouhé sekvence konvenčních obrázků jednotlivých řídce distribuovaných fotoaktivovaných molekul. Ty jsou systematicky lokalizovány se subdifrakční přesností. V této práci jsme navrhli, implementovali a experimentálně ověřili sadu metod pro automatické zpracování, analýzu a vizualizaci dat pořízených jednomolekulovou lokalizační mikroskopií. Tyto metody jsou dostupné ve formě otevřeného softwaru, který jsme nazvali ThunderSTORM. ThunderSTORM se stal jedním z předních softwarů v této oblasti.

Dále představujeme náš návrh nového dvouobjektivového mikroskopu schopného superrozlišení, který zhruba zdvojnásobuje počet detekovaných fotonů. To dále zvyšuje dosažitelné rozlišení násobkem $\sqrt{2}$. Sestavili jsme funkční prototyp tohoto mikroskopu. Dále jsme vyvinuly a experimentálně ověřili metody pro kalibraci a pro analýzu obrazu z tohoto mikroskopu.

V poslední části této práce se zaměřujeme na to, že navzdory vysokému prostorovému rozlišení lokalizační mikroskopie, není tato metoda vždy vhodná pro zobrazování živých buněk z důvodu své špatné rozlišovací schopnosti v čase. Jednou ze strategií je zvýšit hustotu fotoaktivovaných molekul v každém obrázku. Nicméně takový přístup představuje další výzvu při analýze obrazu. Zde prezentujeme 3denseSTORM, nový algoritmus, který je schopný zrekonstruovat 2D nebo 3D obrazy se superrozlišením ze sekvence difrakčně limitovaných obrázků s vysokou hustotou fotoaktivovaných molekul. Tento algoritmus využívá metody komprimovaného snímání a používá Poissonův model šumu, což je velmi důležité v temných podmínkách. Odvodili jsme teoretický limit rozlišení této metody a ukázali obrazové rekonstrukce 2D a 3D dat pořízených simulací i snímáním reálných biologických vzorků. Vyvinutá metoda je vhodná pro rychlou akvizici obrázků hustě označkovaných biologických vzorků, což zlepšuje možnosti studovat živé buňky pomocí jednomolekulové lokalizační mikroskopie.

Klíčová slova

Mikroskopie se superrozlišením, jednomolekulová lokalizační mikroskopie, zpracování obrazu, numerická optimalizace, řídké reprezentace.

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Introduction

A light (or optical) microscope uses visible light (wavelength approximately 400-700 nm), which makes the technique minimally invasive to biological specimens and thus allows for observation of living organisms.

Arguably, the single most important invention in modern cell biology was application of fluorescence and phosphorescence in microscopy. In a fluorescence microscope the specimen is illuminated with light of specific wavelength (energy) which is absorbed by a fluorescent molecule, a fluorophore (or chromophore). This excites an electron of the molecule to higher quantum state. When the electron relaxes to its ground state a photon of lower energy (longer wavelength) is emitted.

The real power of fluorescence microscopy comes from the great contrast which is unprecedented. Since the majority of biological samples are transparent, it can be impossible to distinguish different parts of the cells by transmission or reflection. But with fluorescence, we can label one or more different parts of a cell by different fluorophores with non-overlapping excitation spectra. This way, it is guaranteed that only a particular parts of the cell are observed given the wavelength of illumination.

Fluorescence microscopy has become firmly established as one of the chief tools available for the study of biological systems at the cellular level. Unfortunately, the resolution of fluorescence microscopes

is limited due to wave nature of light (Abbe, 1873). The spot created by diffraction is called the point-spread function (PSF), which is the impulse response of the microscope. The resolution limit is often defined as the smallest separation distance between two point-like objects in which they can still be distinguished as two individual spots. As a result, most resolution criteria are directly related to the properties of the PSF.

As many biological structures within cells are much smaller than the diffraction limit, increasing resolution is of prime importance. Consequently, in last couple of decades the field has been revolutionized and the diffraction barrier has been surpassed by several different techniques (Huang et al., 2009). Imaging beyond the diffraction limit using optical microscopes opens numerous opportunities for biologists to perform analyses that were previously thought impossible. For its great potential super-resolution microscopy was chosen as the Method of the Year by Nature Methods journal (Evanko, 2009). In 2014, the Nobel Prize in chemistry was awarded to Eric Betzig, Stefan Hell, and William E. Moerner for the development of super-resolved fluorescence microscopy.

Single molecule localization microscopy

It has been discovered that when shinning a powerful source of light on a fluorescent sample, some of the fluorophores can be temporarily turned off to a dark state and later return to an emitting state. Thus, theoretically it would be possible to always activate only a single fluorescent molecule at a subregion of a sample so in the recorded image no two molecules overlap. Therefore, the resolution is no longer limited by diffraction. Assuming correct sampling, positions of all molecules in the sample can be recovered with a precision which is only limited only by number of collected photons. More photons mean more accurate localization and consequently higher resolution.

This very principle is the main idea of single molecule localization microscopy (SMLM), which is also known as photo-activated

localization microscopy (PALM) (Betzig et al., 2006), fluorescence PALM (fPALM) (Hess et al., 2006), stochastic optical reconstruction microscopy (STORM) (Rust et al., 2006), direct STORM (dSTORM) (Heilemann et al., 2008) and many more names. The main difference between the methods is in the principle of activation and deactivation of fluorescent molecules. In real experiments, SMLM methods achieve < 20 nm lateral and < 50 nm axial resolution. Since only a subset of fluorophores is active in a single image, typically thousands frames need to be recorded to reconstruct a single super-resolution image. This negatively affects temporal resolution, which is on the order of few minutes, and makes the method largely incompatible with live cell imaging. However, recent progress shows that increasing the density of photoactivated fluorophores opens possibilities for imaging dynamic processes in living cells (Min et al., 2014; Ovesný et al., 2014b).

In recent years, localization microscopy has become one of the most attractive interdisciplinary research areas combining efforts from mathematics, physics, chemistry, and biology. Because this kind of microscopy is computation-heavy, the algorithms for analysis are essential for obtaining any results. This fact sparked research in image processing and data analysis for localization microscopy.

Goals of the thesis

This thesis is concerned with single molecule localization microscopy. SMLM is a super-resolution technique, which typically does not require a complicated setup, therefore, it is relatively cheap to acquire and maintain such microscope. The main burden of SMLM is in analysis of acquired images. For this purpose specialized software is essential. One of the main goals of the project is development of such software to provide a reliable and systematic process of data analysis. Moreover, SMLM suffers from bad temporal resolution. Addressing this issue is also a goal of the thesis. In addition, we aim to design and build a dual-objective microscope, which roughly doubles the number of collected photons and thus further improves both spatial and temporal resolution of STORM images. For this, we need to develop methods for microscope calibration and image analysis.

Materials and methods

Microscopy

Single objective imaging
We used an Olympus IX70 microscope equipped with an Olympus planapochromatic 100× / 1.40 NA oil immersion objective and a NEO sCMOS camera (Andor, Belfast, Northern Ireland). The back-projected CCD pixel size in the sample was 65 nm. A 405 nm, 10 mW diode laser and a 532 nm, 1000 mW DPSS laser (Dragon laser, ChangChun, China) were filtered using bandpass filters and combined with dichroic mirrors (Chroma, Bellows Falls, VT, USA), diffused with a laser speckle reducer (Optotune, Dietikon, Switzerland), then coupled into a 0.39 NA, 600 μm diameter multimode optical fiber (M29L01, Thor Labs, Newton, New Jersey). The fiber output was imaged into the sample using a critical illumination setup. This configuration resulted in an evenly illuminated field. We closed the microscope's field stop so that only a small area of the sample ($\approx$ 30 μm diameter) was illuminated by the full laser power. We isolated Alexa 532 fluorescence using a TIRF filter set (Chroma). For 3D imaging, we introduced a 500 mm focal length cylindrical lens in front of the sCMOS camera (LJ1144RM-A, Thor Labs). Image sequences were acquired using Andor IQ software. We typically recorded 20,000 - 40,000 frames with an exposure time of 100 ms. When acquiring images of densely labeled samples, we typically recorded only 400 frames. For the sake

of simplicity we did not apply any additional corrections for pixel-dependent parameters of the sCMOS camera in any of the evaluated algorithms.

Dual objective imaging
Our design is based on that of Xu, et al. (Xu et al., 2012, 2013), but it is somewhat simpler, while also being easier to align. A schematic, and photographs of the dual-objective microscope are shown in Figure 1. The microscope is equipped with two 100×/1.35NA oil immersion objectives, 405, 473, 532, and 650 nm lasers, and an Andor EMCCD camera. The current sample carrier consists of a micrometer-actuated XYZ stage. The sample is mounted between two high-precision coverslips, then held with rare earth magnets to a piezo-controlled focusing device. This combination allows coarse sample adjustment and focusing followed by nanometer-accurate axial scanning.

Image analysis

Our solution for SMLM data analysis is called ThunderSTORM (Ovesný et al., 2014a). ThunderSTORM is open-source, interactive, modular software, which provides a complete set of tools for automated processing, visualization, simulation, and quantitative analysis of data acquired by SMLM methods including STORM, dSTORM, SPDM, PALM, and fPALM. The software is distributed as an ImageJ plugin (Abramoff et al., 2004), can run on computers with different operating systems, supports the ImageJ macro language, and is also compatible with other ImageJ-based applications such as Fiji (Schindelin et al., 2012) or μManager (Edelstein et al., 2010). ThunderSTORM was developed using a home-built SMLM system, but the software has been tested, and works well with data acquired using commercially available Nikon N-STORM and Zeiss Elyra systems, and offers several unique capabilities compared to the analysis packages offered by

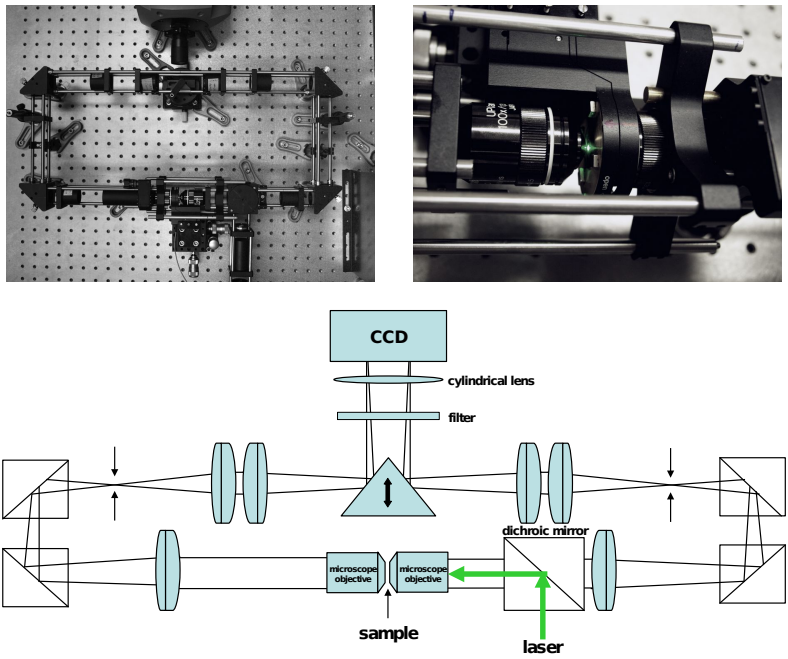

Fig. 1 Dual-objective super-resolution microscope built in our lab. Top: Schematic diagram showing the principal components. Bottom: overview photograph and close-up of the piezo-controlled sample holder with two 100×/1.35NA oil immersion objectives.

these companies. Our philosophy in developing ThunderSTORM has been to offer an extensive collection of processing and post-processing methods which were developed based on extensive testing with both real and simulated data. We also provide a very detailed description of the implemented methods and algorithms as well as a detailed user's guide. ThunderSTORM and the documentation is available at the project website https://github.com/zitmen/thunderstorm/.

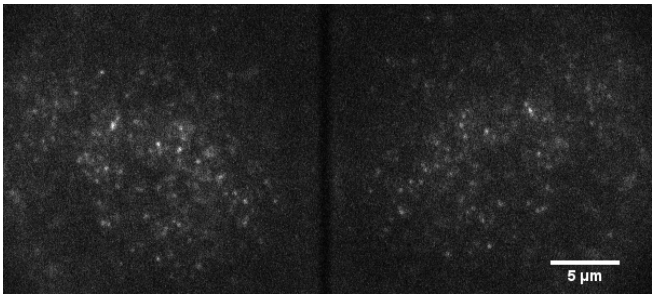

Fig. 2 Example of raw image acquired by our dual-objective microscope with astigmatism.

Calibration for 3D imaging

First, a calibration needs to be performed. The calibration is performed on a stack of images of fluorescent beads of subdiffraction size and high photon emission rate. In case of dual objective imaging (Figure 2), we need to find a geometric transform from one image to the other so the spots can be paired up as images of individual emitters. Finally, defocusing curves are estimated. A defocusing curve specifies how the widths of the PSF changes with varying axial position relative to the focal plane.

Parameter estimation

The approach we adopted in ThunderSTORM can be broken down into several steps, shown as a flow chart in Figure 3.

First, we apply a wavelet filter with kernel generated from a B-spline basis function of the third order with a scaling factor of 2 as suggested in (Izeddin et al., 2012; Starck and Murtagh, 2002). Second, a detector based on non-maximum suppression with wavelet thresholding is applied on the filtered image to find the approximate positions of molecules. Then we use the approximate positions as an initial estimate for maximum likelihood estimation which is performed on the raw input images to determine parameters of PSF with higher accuracy.

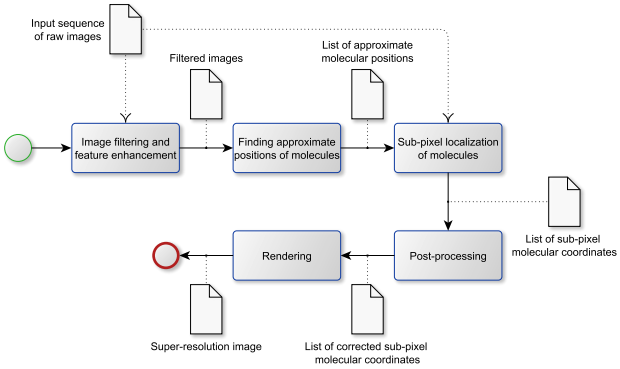

Fig. 3 Data analysis steps for single molecule localization super-resolution imaging. The "pages" represent data or images, while the "boxes" represent processing steps carried out by analysis software.

Finally, the results of estimation can be filtered to remove detections with poor localization uncertainty. Additionally, lateral drift correction or some other post-processing technique is usually applied before the super-resolution image is rendered.

Image analysis of data from the dual objective microscope is similar, but more complex since the raw data consist of images of two focal planes (as an example), one for each objective. Therefore, we need to determine the mapping between the two planes during parameter estimation.

Improving temporal resolution

Single-molecule localization microscopy methods offer high spatial resolution, but they are not always suitable for live cell imaging due to limited temporal resolution. One strategy is to increase the density of photoactivated molecules present in each image. We present 3denseSTORM (Ovesný et al., 2014b), a new algorithm for localization

microscopy which is able to recover 2D or 3D super-resolution images from a sequence of diffraction limited images with high densities of photoactivated molecules. The algorithm is based on sparse support recovery and uses a Poisson noise model, which becomes critical in low-light conditions. For 3D data reconstruction we use both the astigmatism and biplane imaging methods.

The flowchart in Figure 4 indicates multiple steps to localize molecules performed by 3denseSTORM. First, the support of the input signal is determined using a sparse support recovery scheme in a $3\times$ oversampled grid with a 100 nm step in the axial direction. Because the first step introduces a bias towards zero of the recovered molecular intensities, we perform debiasing with a fixed spatial support (Min et al., 2014). This ensures good starting conditions for further processing. Next, approximate positions and intensities of molecules are extracted and continuous refinement is performed by maximum-likelihood estimation according to (Babcock et al., 2012; Laurence and Chromy, 2010). Finally, post-processing can be applied, such as removal of outliers, drift correction, etc.

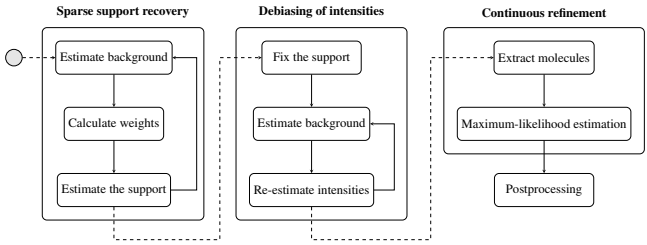

Fig. 4 Flowchart of 3denseSTORM algorithm. Both sparse support recovery and debiasing of intensities are iterative processes. But each iteration is solved by a fixed number of steps since the optimization problems used here all have closed form solutions.

Results and discussions

ThunderSTORM

In 2013 a SMLM challenge was held[1] to evaluate nearly 30 software packages using several different simulated and real sets of SMLM data. We submitted ThunderSTORM for the evaluation. As it turned out ThunderSTORM was declared the overall winner in long sequence analyses (Sage et al., 2015), which was one of two categories of the challenge. To achieve this result we performed a series of extensive Monte-Carlo simulations to evaluate each step of the analysis for different kinds of input data. Here we provide some results where we used ThunderSTORM for real cellular imaging.

An example of z-stage scanning on real data acquired by our single objective microscope and processed by ThunderSTORM is shown in Figure 5. We acquired a total of 9000 frames in 9 planes.

As for another example of cellular imaging, this time with our dual objective microscope, we acquired 1000 frames of a U2-OS cell densely labeled for tubulin. 3D information was extracted from mutual defocus of the microscope's two objectives. Additionally, cross-correlation based drift correction was applied, and the super-resolution image was rendered. The result is shown in Figure 5.

[1] *SMLM Challenge 2013* available at http://bigwww.epfl.ch/smlm/challenge2013/

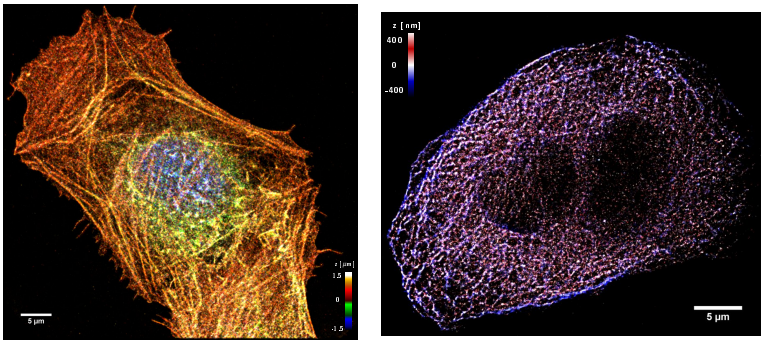

Fig. 5 Left: imaging Atto565-phalloidin labeled actin in U2-OS cells using z-stage scanning. This way, we were able to image deeper into the cell than with just astigmatism. We can see several "layers" in the image, especially in the area of the cell nucleus where the cell is the thickest. Right: imaging of tubulin in U2-OS cells with dual objective microscope.

Improving temporal resolution of 3D SMLM

Quantitative evaluation

We designed Monte-Carlo simulations to quantitatively evaluate the performance of 3denseSTORM in terms of the localization accuracy (lateral and axial), detection rate (F1-score), and recovered molecular density. We generated a series of experiments in which the density of the molecules varied from 0.1 to 20 molecules/μm^2 with a step of 0.5 molecules/μm^2 between each experiment (i.e., 41 independent simulations). The dataset in each experiment contained 100 images 32×32 pixels in size. The pixel size was set to 80 nm and the FWHM of in focus molecules was 260 nm. All molecules were placed randomly inside a central region of 20×20 pixels within an axial range of -400 nm to +400 nm. Each molecule was generated with an integral intensity of 2500 photons. Background offset of 70 photons was added to each image and each such image was additionally corrupted with Poisson noise.

The results were compared to standard single-molecule fitting performed by ThunderSTORM with the default settings and to 3D DAOSTORM (Babcock et al., 2012). All three methods use astigmatic imaging and refinement of localized molecules based on maximum-likelihood estimation. The main difference between these algorithms is the detection method for finding the imaged molecules. The performance of 3denseSTORM was also evaluated for biplane imaging. To demonstrate the importance of accounting for a Poisson noise model, we also show the results for 3denseSTORM with a Gaussian noise model.

Our results indicate that single-molecule fitting is not able to recover densities higher than about 3.5 molecules/μm^2, while 3D DAOSTORM saturates at about 7.5 molecules/μm^2. 3denseSTORM can recover densities up to about 13.5 molecules/μm^2 for astigmatic imaging and up to about 15 molecules/μm^2 for biplane imaging and thus provides the best detection rate compared to other tested methods, see Figure 6. The Gaussian version of 3denseSTORM performed slightly worse than the Poisson version. The estimated theoretical limit was 18.6 molecules/μm^2. It is important to recall that the density limit is directly related to the FWHM of PSF.

Visual examination

For visual examination of the localization accuracy of the detected molecules, we generated a second simulated 3D SMLM dataset with molecules randomly distributed in the shape of a trefoil knot. As in the other simulations, the data consisted of 100 images of size 32×32 pixels. Each image contained 30 molecules, where every molecule was modeled with an integral intensity of 2500 photons. Background offset of 70 photons was added to each image and each generated image was corrupted with Poisson noise. The axial position of the set of molecules was modulated by the generating function for a trefoil knot (a sine function) ranging from -400 nm to +400 nm.

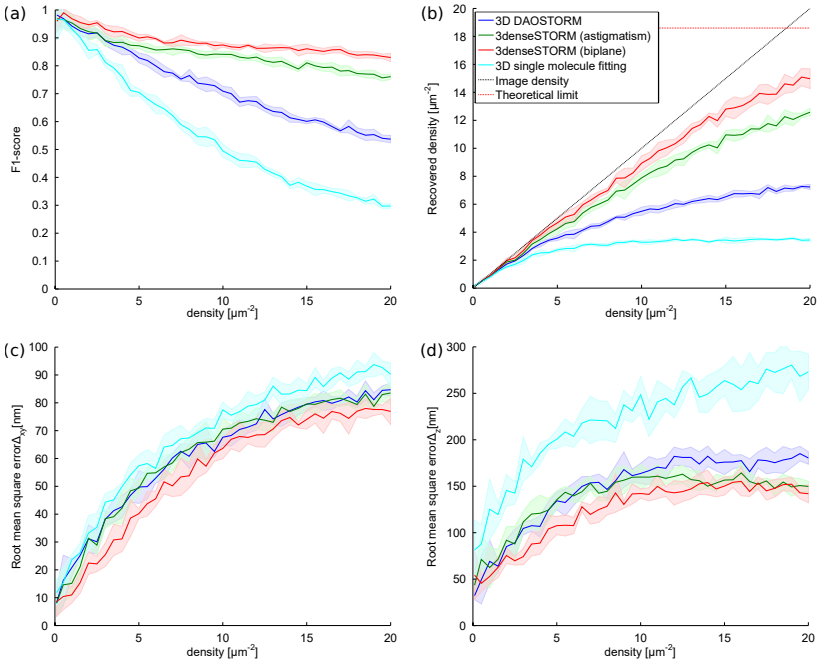

Fig. 6 Evaluation of high density 3D Monte-Carlo simulations. (a) F1-score, (b) recovered density calculated as the number of true-positive detections per μm^2*, (c) lateral localization error, (d) axial localization error. The shaded areas correspond to the standard deviation of five repeated measurements.*

As expected, the low detection rate of single-molecule fitting methods makes the shape of the reconstructed knot incomplete. This is especially noticeable near the intersections, see Figure 7. Moreover, the recovered axial positions of many of the molecules in these areas are not correct. 3D DAOSTORM achieves much higher detection rates and the localization accuracy in the axial direction is better. 3denseSTORM provides the highest detection rate and the localized molecules preserve the shape and continuity of the 3D shape. Also the color-coded axial

position of the molecules is in good agreement with the ground-truth visualization of the simulation.

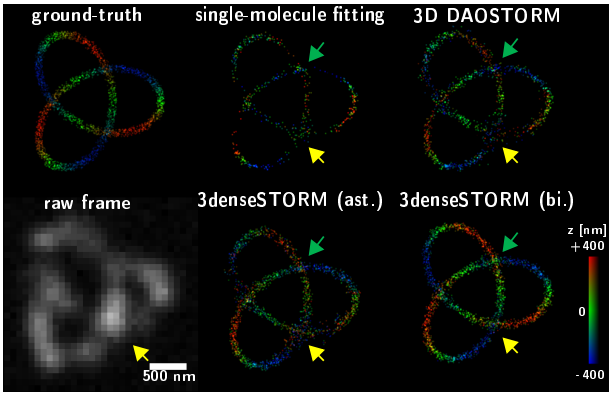

Fig. 7 Trefoil knot. The top panel shows the ground-truth data and the results for single-molecule fitting (astigmatism) and 3D-DAOSTORM (astigmatism). The bottom panel shows an example of one raw data frame and results for 3denseSTORM (astigmatic and biplane imaging).

Real data analysis

To evaluate the quality of 2D image reconstruction with 3denseSTORM, we used a publicly available dataset from the single molecule localization challenge website[2]. The dataset, "Tubulins-high density," contributed by Nicolas Olivier, Debora Keller and Suliana Manley, consists of 500 images of 128×128 pixels. We compared 3denseSTORM with 3D DAOSTORM and with single molecule fitting performed by ThunderSTORM. The results in Figure 8 show that both 3D DAOSTORM and 3denseSTORM reconstruct the data well, even after only 100 frames. Single molecule fitting performed by ThunderSTORM suffers from low detection rates. The reconstructed images produced by 3denseSTORM look sharper in high density areas compared to other

[2] *SMLM Challenge 2013* available at http://bigwww.epfl.ch/smlm/challenge2013/

processing methods, see the region indicated by the yellow square in Figure 8.

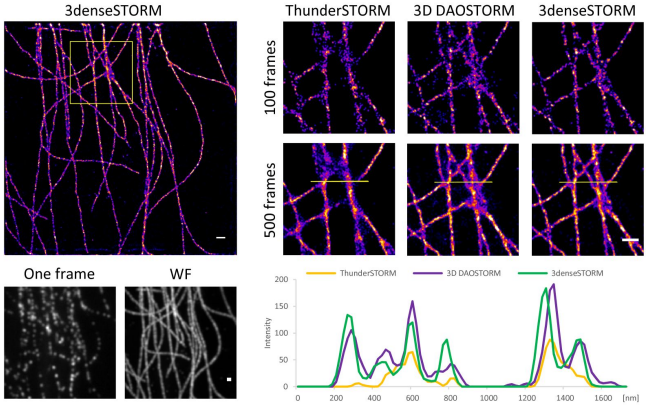

Fig. 8 Comparison of 3denseSTORM, 3D DAOSTORM and ThunderSTORM on real 2D high-density data. The widefield (WF) image was generated as the standard deviation of the entire sequence of 500 frames. The right panel shows a detail of an area marked by the yellow square in the left image. Intensity profiles were measured and plotted from reconstructions of 500 frames. All scalebars are 500 nm.

To further evaluate 3denseSTORM for 3D data reconstruction, we acquired a series of images of immuno-labeled microtubules in U2-OS cells using astigmatic imaging. We analyzed two datasets labeled as ROI 1 (70×70 pixels) and ROI 2 (47×58 pixels), each 400 frames long. We used an exposure time of 100 ms, so each dataset was acquired in ≈ 40 seconds. Both datasets suffer from very low SNR due to high background caused mainly by out of focus fluorescence. The results displayed in Figure 9 show 2D intensity projections to compare how the methods reconstruct sample features. In ROI 1 the arrows indicate areas of high molecular density where 3denseSTORM clearly outperforms the other two algorithms. In ROI 2 the line segments indicate where the intensity profiles were plotted. 3denseSTORM successfully resolved two parallel microtubules while the other methods did not. We also

visualized the result of 3denseSTORM as a 3D image with color-coded z-coordinates in Figure 9.

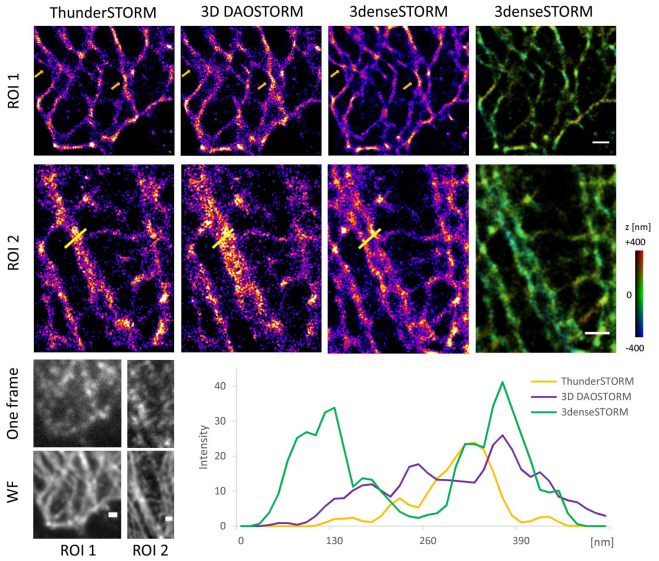

Fig. 9 Comparison of 3denseSTORM, 3D DAOSTORM and ThunderSTORM on real 3D high-density data (U2-OS cells immuno-labeled for tubulin). The widefield (WF) images for each ROI were generated as the standard deviation of the entire sequence of 400 frames. For visual examination, a 2D image for each method was reconstructed. The yellow arrows in ROI 1 mark high density areas. The rightmost column shows a 3D visualization with color-coded z-coordinates. Intensity profiles were measured in reconstructions in ROI 2 and plotted to compare the methods. All scale bars are 500 nm.

Comparison of 3D imaging methods for high-density SMLM

When we analyzed the trefoil knot we encountered an interesting phenomenon upon which we want to expand here. We hypothesize astigmatism isn't well suited for imaging with medium to high molecular densities, because the PSF becomes oriented in a certain direction when

defocusing. As the PSFs start to overlap, this can lead to incorrect parameter estimation when molecules are distributed along an oriented structure like a microtubule. In this case the estimation algorithm tends to distort the PSFs so their orientations align with the underlying structure. This causes a chain effect so parameters of a whole group of molecules are estimated incorrectly. It's worth noting this is certainly not a problem of just our algorithm. We encountered the same issue also with 3D DAOSTORM and others. We believe, this problem is specific to the astigmatic imaging and to the nature of optimization algorithms. Hence, for 3D imaging of dense data we recommend to use a biplane method instead.

To isolate the issue, we generated several datasets of molecules uniformly distributed along horizontal and vertical lines. The axial position of the molecules was varied from -400 to +400 nm. Each dataset contained 10 images with 5 molecules and 10 images of 10 molecules distributed along the lines. Then we analyzed the data with 3denseSTORM. Figure 10 exposes the problem of astigmatism as the results with higher molecular densities show axial positions biased such that elliptical PSFs align with the lines.

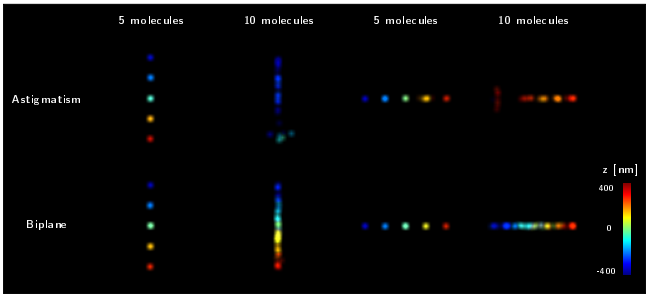

Fig. 10 Comparison of astigmatic and biplane 3D imaging of dense data. Each result consists of 10 averaged measurements. Consider the color bar on the bottom right as an example of ground-truth data. Each of the lines is 1.6 μm *long.*

Conclusions

Super-resolution microscopy is a new attractive and dynamic research field which is already producing impactful results in biomedical research. In this thesis we concentrate specifically on image processing and data analysis for single molecule localization microscopy. We provided a compact overview of SMLM development and discussed all of its aspects, such as fluorophores, microscope design, image processing, and analysis of the results. Below we assess our contributions.

ThunderSTORM

We developed open-source software ThunderSTORM, which introduces several new features and concepts for 2D and 3D SMLM data analysis. The software combines several algorithms for SMLM analysis into one comprehensive environment. We introduce a novel rendering algorithm based on averaged shifted histograms and we implement multiple key improvements to provide the best results possible when imaging in a real-world environment where different kinds of data exist. This was undoubtedly confirmed when ThunderSTORM won the long sequence part of SMLM Challenge 2013, where nearly 30 softwares were quantitatively compared on multiple sets of artificial and real data. Moreover, ThunderSTORM is the only software which provides such a rich repertoire of features for image processing, data post-processing, data analysis, super-resolution rendering, data simulation, and performance evaluation. We currently register over 2600

unique downloads from 57 countries world wide. These numbers were measured by Google Analytics and do not include updates but only first fresh downloads, otherwise the number of downloads would be even higher.

Dual-objective microscope
We built a prototype dual-objective fluorescence microscope, which we successfully used for multiple of 3D data acquisitions. Compared to a conventional fluorescence microscope, the new microscope doubles the count of collected photons and thus further improves resolution in STORM experiments. The design of the microscope is unique. There are only a handful of similar microscopes in the world and this is the only microscope of its kind in the Czech Republic. In addition to the physical device, we also designed a calibration protocol and algorithms for data analysis and we implemented them into ThunderSTORM.

3denseSTORM
We proposed and implemented a novel algorithm, 3denseSTORM, aimed at processing SMLM data with high molecular density. Imaging with a higher density of molecules helps facilitate live cell imaging as data can be acquired much faster, potentially capturing dynamic, 3D movements in the sample. The 3denseSTORM algorithm is capable of processing both 2D and 3D single-molecule data and can be extended for any kind of point spread function. The algorithm utilizes concepts from compressed sensing (CS), which was previously shown to provide unprecedented detection rates. However, compared to previously available methods based on CS, 3denseSTORM is orders of magnitude faster and is more memory efficient. Moreover, it is currently the only CS based algorithm that uses a Poisson noise model in both the molecule detection and position refinement steps. Together, this allows for processing of larger 3D images acquired under the extreme low light conditions which are encountered when imaging single molecules.

The algorithm is robust to high background levels and low signal to noise ratios.

Directions for future research

There are two major areas for future research and development directly coming from the results of this thesis. The first one is to improve the construction of the dual-objective microscope. Specifically the sample holder is not stable enough which causes drift. Also, a different light source or illumination path could achieve more uniform sample illumination. A second area for future research is utilization of temporal information in our algorithm for analysis of images with high molecular density. This seems to be an opportune direction since there is a pressure to apply super-resolution microscopy in live cell imaging. So, we can slowly see that for example localization microscopy and fluctuation imaging start to converge. Although it is worth noting that localization microscopy has many other applications due to its main strength of imaging individual molecules.

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